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Production, purification and properties of *Bacillus macerans* amylase

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PRODUCTION, PURIFICATION AND PROPERTIES
OF BACILLUS MACERANS AMYLASE.

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PRODUCTION, PURIFICATION AND PROPERTIES
OF *BACILLUS MACERANS* AMYLASE

by

Lateef Oyebamiji Emiola

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
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1970

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DEDICATION

To my wife Lola
and to my daughter Olubunmi
for their many sacrifices and
adjustments during the course of this work.

INTRODUCTION

The amylase of *Bacillus macerans* has been of great interest to polysaccharide enzymologists over the years both in terms of its enzymic peculiarities and in the nature of products formed by its action on starch and starchy substrates. *Bacillus macerans* amylase (BMA) is a transglycosylase elaborated extracellularly by *Bacillus macerans*. It differs from other amylases in its unique ability to form cyclic (Schardinger) dextrans from α -1 \rightarrow 4 linked glucosyl polysaccharides. It is the only known enzyme capable of catalysing this unusual reaction. Schardinger dextrans are a group of homologous cyclic polymers of D-glucose containing six or more α -D-glucopyranose residues linked by α (1 \rightarrow 4) glucosidic bonds.

Specifically, BMA has been implicated in three unique types of reactions, namely:

1. A cyclizing reaction in which BMA forms a cyclic product from linear polysaccharide substrates. This naturally demands that the enzyme must be capable of a transferase activity whereby the C-4 hydroxyl group of the non-reducing end glucosyl moiety is coupled to the acetal carbon at C-1 of a glycosyl residue five or more glucose units away.
2. A coupling reaction which involves the attachment of a wide variety of cosubstrates such as D-glucose, maltose, sucrose, panose, sorbose, etc. to the reducing end of the opened cyclic dextrin. It is basically a glycosidic transfer or exchange reaction between the cyclic dextrans (donor substrates) and the cosubstrates (acceptor substrates).
3. The amylase of *B. macerans* has also been reported to be capable

of another type of transglycosylation reaction whereby glycosyl moieties could be transferred from one linear oligosaccharide to another. This is a disproportionation type reaction, otherwise known as "homologizing" reaction.

The physical and chemical properties of Schardinger dextrans have been extensively investigated by several workers, but very little work has been done on the protein characteristics of the enzyme that catalyses the formation of these dextrans from starch. The difficulties involved in culturing *Bacillus macerans*, coupled with relatively long periods of incubation before enzyme production starts may have been one of the reasons for the few attempts that have been made so far to purify BMA.

There are many interesting features of this enzyme which require further scrutiny.

a. For example, the mode of action of BMA has not been clearly worked out. From the data obtained on enzymic action with starch and other polysaccharides, the enzyme appears to be an endoase, *i.e.*, it is capable of a random attack on starch similar to the α -amylases. This appears to be especially true since like α -amylases it quickly renders starch achroic to iodine and degrades starch with a rapid decrease in viscosity. However, the resistance of β -limit dextrin towards BMA attack, argues strongly in favor of an exoase enzyme of the β -amylase type. Further, the involvement of the free C-4 hydroxyl group of glucose (which must occur only on chain ends) as a nucleophile to cleave the α -1 \rightarrow 4 glucosidic bond also demands an exo type mechanism.

b. Water is not known to participate in the enzymic action of BMA on starch; thus it is presently controversial whether or not the

hydrolytic activity that produces reducing sugars along with cyclic dextrans (when crude or moderately purified BMA acts on starch) resides in a contaminating enzyme or it is an intrinsic function of BMA.

c. The nature of the enzymic characteristics permitting the intramolecular transfer reaction leading to the formation of Schardinger dextrans is still a mystery to carbohydrate enzymologists. A knowledge of the active site of this enzyme is therefore desirable.

d. There is disagreement as to the nature of the substrate size requirements for the disproportionation reaction (reaction type 3 above). Cramer and Steinle (1) believed that the presence of cyclic dextrans is required for homologizing reaction to occur, while French (2) has suggested that disproportionation is a concentration-dependent reaction and the presence of cyclic dextrin may not be needed.

Plan and Scope

In order to provide valid mechanistic explanations for the unique reactions involving *B. macerans* amylase catalysis, it is important to re-examine the reactions catalysed by a very pure homogeneous enzyme. The investigations reported in this dissertation have been conducted with the problems outlined above in mind. Thus, the intent of these studies is threefold: (1) Formulation of a suitable medium for the production of BMA in sufficiently high yield and within a reasonably short period of incubation. (2) Exhaustive purification of the crude enzyme. (3) Re-examination of the single reactions catalysed by pure BMA and elucidation of the mechanism of its action on amylose.

SURVEY OF THE LITERATURE

Schardinger Dextrins

Antoine Villiers (3-5) was the first to report the formation of non-reducing crystalline dextrans of the Schardinger type from bacterial digests of starch. In 1891, while investigating the action of representative ferments on carbohydrates under various conditions, Villiers found that his bacterial culture of *Bacillus amylobacter* [*Clostridium butyricum*] degraded potato starch to produce a small amount (0.3%) of nonfermentable, nonreducing crystalline material concomitantly with reducing maltodextrans. Villiers called these secondary crystalline products "cellulosines", because of their apparent resemblance in chemical property to cellulose [for example, they exhibited a marked resistance to acid hydrolysis]. Villiers found that his "cellulosines" recrystallized from water or alcohol to form brilliant crystals with composition represented by a multiple of the formula $[C_6H_{10}O_5]_2 \cdot 3 H_2O$ or $[C_6H_{10}O_5]_6 \cdot C_2H_6O \cdot 5 H_2O$. He also observed that the crystalline dextrans exhibited unique properties as reflected in their unusually high rotatory power, nonreducing effect on copper reagents, complete insensitivity towards phenylhydrazine and extreme resistance to acid hydrolysis. In his opinion, Villiers believed that the formation of these crystalline dextrans from starch was effected by a soluble product secreted by the organism. Koch (6), however, disagreed with this postulate and contended that Villiers used impure cultures. It was Koch's opinion that the spores of *B. amylobacter* would have survived the primitive sterilization procedures employed by Villiers, and that it was possible that his digests contained sufficient amount of

the heat resistant bacterial spores to account for the quantity of crystalline dextrin obtained. This controversy remained unresolved for decades after, because attention was immediately focused on the characterization of the crystalline "cellulosines" produced from starch transformation.

The crystalline dextrans (Villiers' "cellulosines") are today known as Schardinger dextrans in recognition of the great pioneering efforts of Franz Schardinger in this field. Twelve years after Villiers' publications, Schardinger (7) isolated a starch "rotting" organism which produced crystalline dextrans from starch. He designated these crystalline dextrans, A and B, and later observed that his crystalline dextrin B was identical in properties to Villiers' "cellulosine". He subsequently characterized these dextrans as "crystallized dextrin alpha" and "crystallized dextrin beta" (8). The Greek alphabetical designations are still used today, but the crystallized dextrans are now generally known as α -Schardinger dextrin and β -Schardinger dextrin respectively.

For the next twenty-five years after Schardinger's final paper, little real progress was made in the investigations of the crystalline dextrans and their method of formation. The chief investigators of this troubled period were Pringsheim (9) and Karrer (10). Both men and their coworkers used impure materials and placed too much reliance on cryoscopic measurements of molecular weights. Since Schardinger dextrans are of comparatively high molecular weight and are very difficult to separate from low molecular weight impurities (solvent of crystallization and occlusion compounds), use of cryoscopic techniques by these workers led to many anomalous results and bitter arguments between the two groups. Conse-

quently, reports in the literature between 1909 and 1935 are now generally regarded as of little significance. However, a comprehensive review of this period has been prepared by Pringsheim (11), Samec and Blinc (12) and more recently by French (2). The next phase in cyclic dextrin investigation started about 1935 when concerted efforts began on the problems associated with the separation, purification and determination of structure of the Schardinger dextrans. Freudenberg and his school (13-20) as well as French and coworkers (21-24) contributed immensely to our knowledge of the Schardinger dextrans during this period. This era (extending to about 1950) has been appropriately termed "the maturation period" by French (2). Schardinger (8) had earlier characterized the alpha and beta dextrans. Freudenberg and his collaborators (13, 14) provided definitive evidence for the gamma dextrin and suggested that higher homologues existed. Although Freudenberg later on reversed himself on this by questioning the existence of the higher cyclic oligosaccharides; Akiya and coworkers (25-28) in their exhaustive investigations of the cyclic dextrans presented strong evidence for the existence of the higher members.

In the ensuing years significant degree of success was achieved in preparing pure alpha, beta, and gamma Schardinger dextrans by taking advantage of the ability of these compounds to form insoluble complexes with organic molecules. Originally, Schardinger (8) had separated the cyclic dextrans based on the ease of crystallization of the beta dextrin from water and its low solubility at room temperature (1.5%) followed by precipitation of the alpha dextrin from the mother liquor by the addition of alcohol. Freudenberg and Jacobi (13) improved on this method by

devising a separation scheme based not only on solubility differences of the dextrans themselves but also on the differences in solubilities and rates of crystallization of their acetates. Later on, Freudenberg *et al.* (14-16) modified this procedure by exploiting the fact that bromobenzene does not precipitate alpha dextrin, but beta and gamma dextrans are readily precipitated. French and coworkers (23, 24, 29) subsequently worked out an excellent fractionation scheme which avoids acetylation of the dextrans, but makes use of the low solubility of the beta dextrin in water, the differential precipitation of beta and gamma dextrans with bromobenzene and the low solubility of the gamma dextrin in 60% propanol-1.

The successful isolation of pure crystalline dextrans led the way to elaborate studies aimed at providing information about their structure. Although Freudenberg and his collaborators initially considered Schardinger dextrans to be linear molecules with nonreducing end groups, this opinion was later modified (17) in the face of compelling experimental evidences by many workers. By 1950, the cyclic nature of the Schardinger dextrans had been clearly and unequivocally established. Irvine *et al.* (30) started the ball rolling when they obtained 2, 3, 6-tri-O-methyl-D-glucose after exhaustive methylation and hydrolysis of Schardinger dextrans. Freudenberg *et al.* (19, 20) in a later work using a better methylation technique, rigidly identified the same compound which they had obtained in over 90% yield. French and McIntire (21) were able to provide convincing proof that the compounds cannot be open-chain by means of periodate oxidation technique. Freudenberg and Cramer (18) at the same time proposed that flexible ring forms are involved in varying degree in Schardinger dextrans and in starch. By taking advantage of

enzymic specificity, the presence of α -D(1 \rightarrow 4) linkage in Schardinger dextrins was established through preliminary partial acid hydrolysis. Upon subjecting the resultant hydrolysate to sweet potato beta amylase attack, maltose was predominantly obtained, thus verifying that the only linkage present in Schardinger dextrins is α -D(1 \rightarrow 4).

French and Rundle (22) provided confirmatory proof of the structure and molecular weights of alpha and beta Schardinger dextrins by X-ray studies. In subsequent studies with Schardinger dextrins, French *et al.* (31) described the preparation of these dextrins in relatively pure form on a larger-than-laboratory scale. In order to resolve the confusion arising from the use of Greek alphabetical designations for Schardinger dextrins, French and Rundle (22) suggested the usage of the more structurally descriptive nomenclature namely: cyclohexaamylose, cycloheptaamylose for alpha and beta Schardinger dextrins respectively. Shortly after, the degree of polymerization of the smallest members of the cycloamyloses was clearly established to be six, seven and eight for alpha, beta and gamma Schardinger dextrins respectively.

Subsequent investigations on cyclic dextrins were largely on their physical and chemical properties. Excellent accounts of these studies have been reviewed elsewhere (2, 32-34) and will therefore not be repeated here.

Bacillus macerans

Although attention was primarily focussed on the purification and structural determination of the crystalline cyclic dextrins during the maturation period, interest also started to develop slowly but simulta-

neously on the nature of the enzyme that catalyses their synthesis from starch.

In 1903, as part of a study on the processes involved in the bio-deterioration of starch foods, Schardinger (7) became engrossed in certain heat resistant bacterial strains responsible for food poisoning. Later, he succeeded in isolating one of these heat resistant organisms (Strain II) which he had found to transform starch into crystalline dextrins A and B. However, he was forced to temporarily terminate his investigation of the crystalline dextrins because of his inability to maintain a virile culture of Strain II bacteria. A year later (35), he isolated and successfully maintained a culture of a new strain of bacteria that produced acetone by fermentation of carbohydrate media. He found this strain as an accidental contaminant in a sterilized nutrient medium, and named this new microbe *B. macerans* (*macerare*, to rot) because of its rotting action on starch (36). He observed that this new strain not only degraded starch, but also produced crystalline dextrins identical with those he had obtained previously.

The need for a morphologically pure strain became apparent from this initial problems with *B. macerans*, but it was almost two decades later before Zacharov (37) reported the first attempt ever made at a morphological characterization of *B. macerans*. He vividly described an isolation procedure for obtaining *B. macerans* free of other contaminating microorganisms such as *Bacillus subtilis*. He was able to separate the more acid stable *B. macerans* spores from the acid susceptible *B. subtilis* spores by variation of pH conditions.

Porter *et al.* (38, 39) later conducted very comprehensive studies on

the morphological aspects of *B. macerans*. In their studies of the characteristics of sixty-three freshly isolated strains and authentic cultures of sporulating, facultative bacteria that produce gas from carbohydrate media, two strikingly distinct groups of aerogenic bacilli were found: the "macerans" groups of which *B. macerans* is typical, and the "polymyxa" group represented by *B. polymyxa*. Among the identifying features of the former group of organisms are their negative "Voges-Proskauer" reaction; production of acid and gas from sorbitol and rhamnose media; exhibition of optimal growth between 42°C to 45°C and relative dormancy at 20°C. They found their *B. macerans* strain to be physiologically and serologically homogeneous.

Bacillus macerans Amylase

Villiers (5) in his studies with *Bacillus amylobacter* ferments, postulated the existence of a soluble product in the culture medium of the bacteria that is capable of the transformation of starch into the crystalline dextrans even in the absence of the organism. He had claimed then, [in spite of vehement objections from Koch (6)] that the soluble substance was being formed continuously in very small amounts and "exhausted as rapidly as it is formed".

Some four decades later, workers in this field began to reassess Villiers' claim. Their subsequent recognition of the catalytic nature of this starch-splitting substance led many investigators to direct efforts toward the establishment of optimal conditions required for the elaboration of Villiers' "soluble product". Freudenberg and Jacobi in 1930 (13) reported their experimental findings on ways to obtain effective

cultures of *B. macerans* and recommended growing the organism on a transfer cycle involving ten days each on meat extract; potato slurry- CaCO_3 ; and potato wedges. They obtained highly virile bacteria after repeating this cycle three times.

The first report of the isolation of an enzyme from *Bacillus macerans*, that was capable of converting starch into cycloamyloses, was not made until 1939. Tilden and Hudson (40) in studies directed towards providing a definitive answer to the question whether or not the crystalline dextrans represent comparatively simple components of starch, or whether they are formed as the result of synthetic activity of the living organism, demonstrated that sterile filtrates from *Aerobacillus macerans* cultures contain an enzyme which converts gelatinized starch into a mixture of the nonreducing alpha and beta Schardinger dextrans. They called this new enzyme *Bacillus macerans* amylase (BMA). The organism was initially cultured on a medium composed of 10% sliced raw potato and 1% calcium carbonate; however, they later found (41) that a 5% rolled oats medium buffered with 2% CaCO_3 was most suitable for maximal enzyme production. Shortly after Tilden and Hudson's report, Freudenberg and coworkers (42) stated that they too had been able to observe enzyme activity in the filtrates of *B. macerans* cultured on yeast extract. Blinc, (43) a couple of years later reported an improved procedure for obtaining optimal enzyme yield based on modification of the Freudenberg's transfer cycle. Blinc also established the optimum temperature of 40°C for inoculation and 45°C for incubation of the organism.

The first systematic effort at improving the production of *Bacillus macerans* amylase was made by Daniels and Stahly (44). They found that it

was possible to increase the yield of enzyme and to decrease the period of incubation from a range of two/three weeks to a period of seven days by ample provision for aeration and oxygenation in the growing culture. Kneen and Beckord (45) at about the same time demonstrated that maximum and very high yields of *macerans* amylase system can be obtained in four days at 35°C using wheat bran extract supplemented with peptone, phosphates, and calcium carbonates. Liggett and Mussulman (46) later accomplished a significant decrease in the period of incubation to three to seven days by supplementing the oatmeal-calcium carbonate medium with ammonium ions. In a modification of the original Tilden-Hudson rolled oats-calcium carbonate medium by employing sufficient aeration, ammonium ions, and trace elements, Schwimmer and Garibaldi (47) reported a ten-fold increase in enzyme yield and a remarkable reduction in incubation time to ten to twelve hours.

Until recently when DePinto and Campbell (48) isolated an intracellular preparation of *macerans* amylase, the enzyme used by previous workers were sterile extracellular filtrates. In 1964, DePinto and Campbell demonstrated that highly active *Bacillus macerans* amylase is elaborated intracellularly by *B. macerans* when cultured on a starch-casein hydrolysate-yeast extract medium at 38°C. Two different enzymic systems were isolated within eighteen to twenty-four hours by these investigators. They have given the names *Bacillus macerans* amylase to the enzyme which converts starch to cyclic dextrins; and *Bacillus macerans* cyclodextrinase to the preparation demonstrated to hydrolyse cyclic dextrins and starch to reducing sugars and linear oligosaccharides respectively. The presence of this latter enzyme had earlier been

speculated by Kneen and Beckord (45). Since these investigators observed no enzyme activity in their eighteen hour extracellular filtrate, they concluded that the detection of BMA in culture filtrates by previous investigators was probably due to lysis occurring during long incubation periods. No other group of investigators has so far corroborated this assertion. Therefore, the locus of elaboration of BMA is currently a subject of much controversy.

As a logical sequel to the successful isolation of *macerans* amylase, Tilden *et al.* (49) reported the first attempts on the purification of this enzyme. Their purification procedure involved a series of fractionation and precipitation steps including treatment with acetone, adsorption on aluminum hydroxide in presence of acetate buffer at pH 4.8 and subsequent elution of the enzyme with phosphate buffer at pH 7.6. Further concentration with acetone followed by dialysis against water resulted in an enzyme product with a 140-fold increase in activity over the initial crude enzyme. Other workers (50, 51) have reported obtaining a substantially purified enzyme by subjecting *B. macerans* culture filtrate to a series of organic solvent fractionations, starch column chromatography, and ammonium sulfate fractionation. Schwimmer and Garibaldi (47) as well as DePinto and Campbell (52) have independently demonstrated that their purified enzyme was electrophoretically homogeneous and almost free of any hydrolytic enzyme contaminant. However, further purification is still regarded as essential, since action pattern studies of the purified enzyme by these workers still show significant hydrolytic activity (47, 53).

In preparing the pure enzyme it is desirable to monitor the activity of the enzyme solutions at each step in the purification. Quantitative

investigations of the biochemistry of BMA activity have been severely handicapped by the lack of a simple and reproducible assay method. A number of methods of assaying have been reported in the literature. Comparison of the specific activity of the purified enzyme from various workers has been difficult because of the very wide range of tests and activity units in use. The Tilden and Hudson approach (41) was based upon Schardinger's original observation that alpha and beta dextrans form crystalline compounds with iodine. Tilden and Hudson, therefore developed a microscopic slide test which measures time of formation of crystalline cyclohexaamylose-iodine needles. Apart from the time-consuming nature of the test, this method suffers from the lack of precision necessary for accurate research and the fact that enzyme activity is slowed but not completely stopped by addition of iodine reagent. However, it is the only known assay that is specific for the *macerans* amylase activity, and some improvement in quantitation is desirable here. An alternative method based on colorimetric measurement of the rate of loss of iodine color of a starch solution was announced in 1951 by Hale and Rawlins (50). While this method is feasible from the vantage of time, it is analytically objectionable because of curved and not often reproducible calibration plots coupled with the inability of the test to identify between hydrolytic and transglycosylase-(Schardinger dextrin forming) activity. An elegant quantitative method was suggested from the work of Schwimmer and Garibaldi (47). This test depends on availability of rather large amounts of pure beta-amylase and it involves measurement of the degree of susceptibility of BMA digests of a given substrate to beta-amylase attack. Although this appears to be a promising method, its major demerit is

that it is not discriminative between cyclising and hydrolytic activities. An additional drawback is the requirement of large amounts of pure glucamylase-free beta-amylase. French and his collaborators (54) developed a method of assay based upon the optical rotational shifts associated with coupling reactions of *macerans* amylase. The chief disadvantages of this method of assaying are (1) the low values of the rotational shifts and (2) the nonspecificity of the assay since it cannot distinguish between rotational shifts arising through the coupling reactions and that due to secondary effects such as mutarotation of the D-glucose co-substrate, and hydrolytic enzyme contaminants. DePinto and Campbell (48) introduced a modified dextrinogenic assay based on the ease of hydrolysis of BMA digest by mineral acid. This method is not very sensitive because of the significant resistance to acid hydrolysis which is characteristic of the cycloamyloses. Thoma *et al.* (55) assayed for *macerans* amylase activity by taking advantage of the coupling reaction that the enzyme catalyzes between cyclohexaamylose and an acceptor like methyl- α -D-glucoside. The resulting methyl- α -D-maltoheptaoside is then rapidly hydrolysed by an excess of added hydrolase (*e.g.*, glucamylase). The amount of glucose formed is determined by reducing value or other appropriate analytical methods. Although this test is more quantitative than others mentioned above, it has certain drawbacks in precision work. (1) The presence of contaminating cyclodextrinase as well as significant trace quantities of acceptors in BMA preparations often results in excessively high blank readings. (2) The requirement of an auxiliary enzyme together with its inherent limitations constitute a major practical objection of this method of assay.

Despite all these technical and operational difficulties in method

of assaying, enzymic preparations with a reasonable degree of purity have been obtained by many workers.

Many investigators have studied the properties of BMA as a protein using enzyme preparations of varying degrees of purity. Evidence for the large size of the enzyme molecule first came from Hudson's laboratory (49). Tilden *et al.* observed that their purest BMA preparation passed through a colloidon membrane of 60 nm porosity, but was retained by a 40 nm membrane. This indicated that each molecule of *macerans* amylase must be of relatively large size. Schwimmer and Garibaldi (47) later reported a minimum molecular weight of 54,000 for their ten-fold purified enzyme. On the basis of sedimentation and electrophoretic studies Schwimmer (56) later speculated that the molecular weight of BMA must be close to 100,000. DePinto and Campbell (52) in a recent publication reported a molecular weight of 139,000 based on calculations from sedimentation and diffusion data.

Typical environmental effects on enzymic activity have been reported. Tilden and Hudson (40, 41) found an optimum temperature for enzyme action at 40°C, and a pH range of maximal activity between 5.6 to 6.4. They also observed that the enzyme is relatively heat stable and no marked inactivation occurred for a period of one hour at 50°C. Schwimmer and Garibaldi (47) in an extensive study of the properties of *macerans* amylase, found that their purified enzyme was stable in iodine solution at pH values less than 4.7 but unstable at pH values above 6. This situation was reversed when the enzyme was changed to an aqueous environment. Since iodine in acid solution (pH 3-5) reacts specifically with the sulphydryl groups of proteins, and with phenolic hydroxyl groups in neutral solutions (57),

these workers were prompted to conclude that BMA probably requires a tyrosyl group for activity instead of a sulfhydryl group. Schwimmer (56) in a later investigation of the properties of his 60-fold purified enzyme found among other things an isoelectric point of pH 4.5, and a turnover number of 43,000 for the electrophoretically homogeneous protein. Some hydrodynamic and kinetic parameters of *macerans* amylase were recently determined by DePinto and Campbell (52) who reported a partial specific volume of 0.712 ml/g, a frictional ratio of 1.79, E_{280} of 9.9×10^4 , a K_M starch of 3.33×10^{-3} g/ml, a pH optimum of activity between 6.1 to 6.2, and an energy of activation in the temperature range 25°C to 55°C of 12,250 calories/mole for their purified enzyme. The relatively low isoelectric point of pH 4.5 determined by Schwimmer (56) was given further support by DePinto and Campbell's amino acid analysis of *macerans* amylase. They found a ratio of 2.6 for $\frac{(\text{aspartic acid} + \text{glutamic acid})}{\text{lysine} + \text{arginine}}$. No cystine or cysteine was obtained, and the anthrone test for bound carbohydrate was negative.

Many features of BMA have been enigmatic to the early workers. The nature of the products formed from enzymic action on starch has been a matter of speculation for many years. The enzymic properties which permit the interesting reactions of this enzyme are still a mystery until today. As a natural sequel to the purification of *macerans* amylase and the consequent elucidation of the structure of the Schardinger dextrans, considerable speculation arose from time to time as to the mechanism of action of BMA and the significance of the reaction in respect to the chemistry of starches and other polysaccharides. Initially, there was lack of agreement as to whether cycloamyloses were starch degradation products, transforma-

tion products or products of bacterial metabolism. These divergent viewpoints generated heated debate among workers in this field during the periods prior to and immediately following "the maturation period".

Freudenberg (58) at first held the view that the crystalline dextrins were preformed in starch because of their iodine staining ability, and he postulated (42) that *Bacillus macerans* amylase splits helical starch chains into cyclic fragments containing five or six glucose residues. Tilden and Hudson (40) also at first thought that the cyclic dextrins are either true components of starch, or are closely related to such true components because of their relative ease of formation from starch without liberation of glucose or maltose. Samec (59) approached the problem from a different angle by assuming one of two hypothesis was true: (1) "that cyclic structures are preformed in the starch molecule" or (2) "that *Bacillus macerans* amylase has a synthetic as well as a degradative action".

Subsequent investigations of *macerans* amylase action on different amylaceous substrates showed that the cyclic dextrins are indeed products of enzymatic synthesis.

Many investigators have noted that the total yield and relative proportions of the alpha and beta dextrins vary with different starchy substrates. French *et al.* (23, 24), and McClenahan *et al.* (60) have separately shown that the relative proportions of alpha and beta dextrins can be altered by the presence of a precipitant. The yield of Schardinger dextrins from *macerans* amylase action on starch has been reported by Hudson and coworkers (49, 61) to be up to 55% of the original starch substrate.

Kerr (62, 63) noted that the yields of the Schardinger dextrins were higher from the amylose fraction of starch than from the amylopectin

fraction. Wilson *et al.* (61) in an independent investigation obtained identical results. Other workers (45, 64) have reported that during the enzymolysis of starch with BMA preparations, the reducing values and amounts of fermentable sugars gradually increase and that the total yield in Schardinger dextrans rises to a maximum and then drops almost to zero. Cramer and Steinle (1) extended these observations using purified *macerans* amylase with very dilute (0.05%) amylose. The initial reaction gave almost entirely alpha dextrin, but as reaction progressed, increasing amounts of the beta and gamma dextrans were produced together with substantial amounts of reducing oligosaccharides. They reported a final yield of over 90% total Schardinger dextrans. Kerr (62) was the first to demonstrate that acid modification of corn starch resulted in yields of Schardinger dextrans that vary inversely with the degree of preliminary hydrolysis. Later on Norberg (51), Cramer and Steinle (1), Samec and Cernigoj (59) and Levine (65) independently showed that very short chain maltodextrans are converted by BMA into cycloamyloses. From investigations by Samec and Cernigoj (59) it was clearly established that the yield of Schardinger dextrans from starch modification or degradation products are in general very low. Data from subsequent studies by several investigators showed that cyclic dextrin formation by BMA is greater in linear (amylose-type) starchy substrates, than in branched (amylopectin-like) substrates.

The large differential in rate and yield of cyclic dextrans between linear and branched substrates generated a lot of speculations about the way BMA act on its substrates. The characterization of the mode of action of BMA has presented a formidable challenge to early polysaccharide enzymologists. It was originally believed that the action of this enzyme

was essentially that of degradation of starch in the same way that beta amylase degrades starch. Action of *macerans* amylase (like beta amylase) was assumed to begin at the non-reducing terminal of a straight chain or exterior chain of a branched substrate. Schardinger dextrans are then produced until the enzyme comes to the end of a straight chain substrate, or a branching point in the case of a branched compound. Evidence for an exo-type of attack of this kind was first suggested by Myrbäck and Gjörling (64), who found that BMA pre-treated samples of starch produced little or no maltose upon subsequent treatment with beta-amylase. Comparative studies of maltose and Schardinger dextrin production revealed to these workers that a decrease in maltose production occurs in those samples which had produced the greatest amount of Schardinger dextrans. They concluded from these observations that BMA attacks preferentially those linkages susceptible to beta-amylase action; *i.e.*, the straight chain fraction of starch and the non-reducing end chains of amylopectin and glycogen. Samec (59) had earlier reported similar observations in their studies with beta limit dextrans. Shortly after Myrbäck and Gjörling's studies, Norberg (51) demonstrated that *Bacillus macerans* amylase effected a rapid decrease in viscosity without the formation of any detectable cyclic dextrans or reducing sugars, when allowed to react extensively with beta limit dextrans.

Although *macerans* amylase has not been clearly established as a hydrolase, certain aspects of its action are analogous to that of alpha-amylases. BMA preparations have been shown by many investigators (43, 60) to exhibit activity with a remarkable resemblance to an endo-type mechanism of attack. Starch is rapidly rendered achroic to iodine, and

low molecular weight products are produced as a direct result of its action on starch. Blinc (43) was the first to observe that the mode of action of BMA upon amylose is characterized by a fast drop in viscosity during the initial stages in enzymolysis. He concluded that the enzyme appeared to behave like an alpha-amylase producing initially high rotatory primary degradation products, which then undergo secondary reactions such as ring condensation. McClenahan *et al.* (60) confirmed this early observation by Blinc, when they reported that their purified *macerans* amylase acted on starch substrates with an initial rapid decrease in viscosity and a gradual decline in optical rotation.

While the intramolecular transfer reaction leading to cyclic dextrin formation remains a distinctive feature of BMA, this enzyme has also been reported to participate in a number of other reactions. Levine (65) first observed that *macerans* amylase converted alpha dextrin into beta dextrin in the presence of certain cosubstrates such as maltose, sucrose, fructose, and glucose. The involvement of *Bacillus macerans* amylase in coupling reaction has been documented by other workers by isolation of coupled products as well as by analytical chromatographic experiments (54). The acceptor specificity of *macerans* amylase in coupling reaction is now known to be unusually broad. Along with those cosubstrates mentioned above, other well characterized effective acceptors are D-glucoheptulose, maltobionic acid, sorbose, turanose, cellobiose, planteose, melezitose, panose, polygalitol, isomaltose and various aldohexoses and aldopentoses. More recently, investigations by Wheeler *et al.* (66) have shown that structural modification of glucose at position six does not prevent coupling. They demonstrated that neither C-6, C-1, nor C-2 hydroxyl groups

nor their stereochemistry are critically implicated in binding an acceptor to the enzyme. As a result D-xylose, 6-O-methyl-D-glucose, and 2-deoxy-D-glucose are suitable acceptors. The most active cosubstrates found, however, are those having unmodified glucose structure. Changes at C-3 and C-4 prevents coupling reaction, so that 3-O-methyl-D-glucose, and D-galactose are not acceptors. The failure of D-galactose to couple with the donor substrate (cycloamyloses) has been cited as a strong evidence for the involvement of the C-4 hydroxyl group in coupling reaction. It has now been clearly established (24, 67) that the action of *Bacillus macerans* amylase is reversible.

In a concerted effort to provide explanations for the conversion of cyclohexamylose to cycloheptaamylose, Levine (65) postulated that coupling reaction leads to formation of linear maltooligosaccharides which are in turn converted to cyclic dextrans plus linear chains of different length. Occurrence of reactions of the latter type in which maltodextrins are disproportionated has been confirmed by several workers (54, 67). Norberg and French (67) showed that maltoheptaose is redistributed by *Bacillus macerans* amylase to give higher and lower oligosaccharides. Pazur (68) repeated similar investigations by monitoring the reaction with paper chromatography and he found simultaneous appearance of all types of linear maltodextrins. He proposed that disproportionation occurs by a random process. Cramer and Steinle (1), however, held a different view on this. Working with very low substrate concentrations of maltohexaose and maltoheptaose of about one per cent (as opposed to 5-10% used by Pazur), they observed no evidence for rapid disproportionation and therefore suggested that cyclic dextrans are essential products or reactants in homologizing

reactions. This controversy has so far remained unresolved.

In retrospect, although much work has been done in an effort to elucidate the mechanism of action of BMA, some finer points of detail are yet to be explained, *e.g.*,

1. While the intramolecular transfer reaction leading to torus formation stands as a characteristic feature of BMA, the conformational properties of the enzyme which brings this unique reaction about is still undetermined.

2. The nature of the substrate(s) participating in the homologising reaction remains a moot point.

3. The classification of BMA as an endoase or an exoase must await further investigations on the mode of action of very pure enzyme on different starchy substrates.

EXPERIMENTAL INVESTIGATIONS

Materials

Organism

B. macerans strain ATCC 8517, a protease-negative mutant, was originally isolated by Maqsd and Robyt¹. Stock cultures of this organism were maintained on potato agar slants and stored in the cold below 10°C.

Culture medium

The organism was grown in an aqueous culture medium which contains 0.5% maltodextrin (DP \approx 44) obtained from *B. subtilis* α -amylase digest of starch (69), 0.01% barbituric acid, 0.10% bacto-peptone, 0.2% $(\text{NH}_4)_2\text{SO}_4$, 0.05% urea, 0.15% yeast extract and 1% CaCO_3 . The pH was maintained between 6.8 and 7. Usual sterilization in the autoclave was at 121°C and 15 pounds pressure for 30 minutes.

Reagents

All reagents used unless specified otherwise were laboratory reagent grades.

Gel filtration chromatography and ion exchange chromatography

Sephadex gels were obtained from Pharmacia Fine Chemicals Inc., Piscataway, New Jersey. Bio-Gel P-150 was purchased from Bio-Rad Laboratories, Richmond, California. Gels were prepared in accordance with the manufacturer's instructions.

¹Maqsd, A. and Robyt, J. Unpublished work. Department of Biochemistry, Iowa State University, Ames, Iowa. 1970.

Electrophoresis

Disc gel electrophoresis apparatus was a product of Canal Industrial Corporation (Canalco), Bethesda, Maryland. The polyacrylamide gel as well as the required buffers were made up in accordance with the 1965 Canalco formulation sheet. A 400V range Heathkit regulated power supply was used to generate power.

Paper chromatography and radioautography

Whatman 3MM chromatography paper was used for all chromatographic analyses. Radioautograms were made by exposure of properly irrigated chromatographic paper to Kodak no-screen medical X-ray film NS-54T.

Substrates

Schardinger dextrans Cyclohexaamylose, cycloheptaamylose and cyclooctaamylose were prepared according to the scheme described by French and co-workers (23) involving use of selective organic precipitants for separating and purifying the cyclic dextrans.

Amylose A sample of recrystallized Superlose (a commercial amylose preparation from Stein-Hall and Co., New York) prepared according to Robyt and French (70) was available in the laboratory. This material was used without any further purification.

Amylopectin Amylopectin was prepared by Hopkin's procedure (71) involving cyclohexanol-thymol precipitation of amylose from potato starch.

β -Amylase limit dextrans Shellfish glycogen β -limit dextrin was prepared according to the procedure outlined by Brammer (72) who kindly donated the sample used in this study.

Amylopectin β -limit dextrin was obtained by exhaustive action of β -amylase on waxy maize starch along the procedure outlined by Robyt and French (69).

Glycogen A three times reprecipitated sample of shellfish glycogen from Sigma Co., St. Louis, Missouri was available in the laboratory and was used without any further treatment.

Radioactive compounds UL- ^{14}C -glycogen was a gift from Mr. Chiharu Nakai of Iowa State University. UL- ^{14}C -starch was purchased from International Chemical and Nuclear Corporation, City of Industry, California. Uniformly labelled amylose was isolated from a 15-hour pullulanase digest of UL- ^{14}C -starch by means of successive gel filtration chromatography on Sephadex G-25 and G-100.

Enzymes

B. subtilis α -amylase was a crude enzyme preparation (HT-concentrate) purchased from Miles Chemical Company, Clifton, New Jersey. It was purified and recrystallized three times before use according to the scheme developed by Robyt¹.

Pullulanase from *Aerobacter aerogenes* was isolated by the method of Bender and Wallenfels (73) as modified by Abdullah *et al.* (74). This preparation showed no α -amylase activity.

Crystalline sweet potato β -amylase prepared according to Balls *et al.* (75) was purchased from Worthington Biochemical Corporation, Freehold, New

¹Robyt, J. F. Unpublished work. Department of Biochemistry, Iowa State University. Ames, Iowa.

Jersey. It was rendered free of glucamylase contamination according to the procedure developed by Katar and Robyt¹.

Analytical Methods

Standard assays

Two different assay methods were employed for determining enzymatic activity of BMA.

Tilden and Hudson microscopic slide test (41) This is a semi-quantitative assay. Activity was determined by adding 0.50 ml of properly diluted enzyme preparation to 1 ml of 3% Merck's soluble starch at pH 6. The solution was then incubated at 40°C, and three-drop aliquots were transferred at varying time intervals to a spot plate. A drop of 0.1N iodine in 0.1M potassium iodide was added and thoroughly mixed in with a wire loop. A loopful of the mixture was then streaked on a microscope slide. Examination of the streak under the microscope after evaporation of the solution revealed the blue hexagonal structures characteristic of α -dextrin-triiodide crystals. The stage when the color becomes brown-violet was used as a suitable end point. From the time (in minutes) required to reach this point was calculated the Tilden and Hudson unit of activity for the enzyme:

$$\text{Unit} = \frac{30 \text{ minutes} \times F}{\text{minutes conversion} \times 0.5}$$

where F = dilution factor of enzyme. One unit of activity is defined as the amount of enzyme required to hydrolyse 30 mg of starch (1 ml of 3%

¹Katar, M. and Robyt, J. F. Unpublished work. Department of Biochemistry, Ames, Iowa.

starch solution) to the brown-violet end point in 30 minutes at 40°C and pH 6.0.

Cyclodextrin transglucosidase assay This is a rapid and more precise method for estimation of BMA activity. The technique takes advantage of the ability of BMA to transfer a cyclodextrin to a suitable acceptor and generate thereby a linear substrate susceptible to hydrolytic cleavage by an appropriate hydrolase. The original procedure described by Thoma *et al.* (55) was slightly modified for the studies reported here. Glucamylase (Dextrinase 80, purchased from Miles Chemical Company, Clifton, N. J.) was used as the auxiliary enzyme instead of the α -amylase used by Thoma and co-workers. Standard assay conditions were as follows:

Solution A: 18 mg/ml cyclohexaamylose, 4 mg/ml α -methyl-D-glucoside, 1.2 mg/ml NaCl, 6.12 mg/ml β -glycerophosphate pentahydrate (a disodium salt preparation obtained from Sigma Chemical Company, St. Louis, Missouri). At pH value of 6.5 this solution remains stable for months when stored below 10°C.

Solution B: 2.5 mg/ml glucamylase, 6.12 mg/ml β -glycerophosphate pentahydrate. Solution B was usually stored cold at pH 6.5 for maximum time of about three to four weeks.

After incubating each component separately at 40°C for about ten minutes, assay was initiated by adding 0.5 ml of the appropriately diluted enzyme solution (enzyme diluted with 20 mM glycerophosphate buffer at pH 6.5) to the mixture of 0.5 ml solution A and 0.1 ml of solution B. The reaction was allowed to proceed for ten minutes at 40°C, and was then stopped by boiling for 10 to 15 minutes in a boiling water bath. Blanks

of enzyme solution alone, and solution A and B combined were similarly treated. Reducing values were measured by the alkaline ferricyanide-cyanide method (76) with the Technicon Autoanalyzer. To obtain a standard curve, the percent transmittance of each standard was plotted on semilog paper (1 cycle \times 70 divisions) versus the concentration of maltose per milliliter. The concentration of apparent maltose in the assay digest was determined from the standard curve. On this basis, the activity converts directly into International units. This was calculated as follows:

$$\begin{aligned} \text{Unit} &= \frac{C \times f_1 \times f_2 \times f_3}{M \times 10 \times 6} = \frac{C \times f_1 \times f_2 \times f_3}{342 \times 60} \\ &= \frac{C \times f_1 \times f_2 \times f_3}{2.052 \times 10^4} \end{aligned}$$

where C = micrograms apparent maltose/ml/10 minutes as read from standard curve.

M = molecular weight of maltose = 342.

f_1 = dilution factor of the enzyme preparation.

f_2 = dilution factor of the digest.

f_3 = total volume of the digest.

The factor 10 in the denominator is the number of minutes the digest was incubated, and the factor 6 is the number of reducing end equivalents obtained per one equivalent of the cyclohexaamylose substrate coupled by BMA to the acceptor.

Protein determination

The concentration of protein at each step in the purification process was measured by the method of Lowry *et al.* (77) with the Technicon Autoanalyzer using bovine serum albumin as standard. For monitoring

effluents from chromatographic columns, measurement of absorbance at 280 nm was found more convenient using Beckman model DU Spectrophotometer with a Gilford Instruments digital readout absorbance meter attachment.

Determination of radioactivity

Radioactivity was measured in a Packard Tri-Carb Liquid scintillation spectrometer model 3000 series. The following liquid scintillators were used:

For toluene-miscible or suspended sample, 3 g/l PPO (PPO = 2,5-diphenyloxazole) and 0.1 g/l POPOP (POPOP = 1,4-bis-[2-(5-phenyloxazolyl)]-benzene in toluene.

For aqueous solution, 10 g/l PPO, 0.25 g/l POPOP, 200 ml ethanol, in dioxane.

Production of *B. macerans* Amylase

Culture conditions

As precultures, 25 ml of sterilized nutrient medium in 250-ml Erlenmeyer flasks were inoculated from the stock cultures of *B. macerans* ATCC 8517 protease negative mutant. After three to four days incubation at 40°C with shaking, 10 ml of this actively growing culture was transferred into 100 ml of freshly sterilized nutrient medium in 500-ml Erlenmeyer flasks. These were incubated for an additional three to four days at 40°C with shaking. For small scale enzyme production, these precultures could be filtered to obtain active enzyme at either stage of the preculturing.

For large scale enzyme production, 100 ml of the second stage culture was used as inoculum for 900 ml of freshly sterilized medium. After subsequent incubation for three days at 40°C, the entire 1 liter batch was

transferred into 9 liters of fresh medium in a 14-liter fermentor assembly (Microferm Laboratory Fermentor, New Brunswick Scientific Company, N. J.). Agitation was at 200 rpm and aeration was at the rate of 1 liter per minute. The temperature of propagation was maintained at $40^{\circ}\text{C} \pm 1^{\circ}$. Two to three additions of maltodextrin (DP44) were often added at 24-hour intervals to keep the level of carbon source at 0.5%. The synthesis of BMA begins after a lag of about twenty-two hours. Approximately 1.0 Tilden and Hudson unit of enzyme per milliliter of liquor was present after two days and the amount rapidly increased between the third and fifth day. The course of enzyme development in several production runs using different carbon sources is shown in Table 1 under results. Subsequent refills of the fermentor were accomplished by addition of 9 liters of fresh medium for every 9 liters of active culture withdrawn for enzyme purification.

Purification of *B. macerans* Amylase

Isolation

At the completion of fermentation, the bacterial cells and residual debris were removed from the fermented medium by means of a supercentrifuge. This was achieved by transferring the entire 10-liter liquor into a reservoir that feeds directly into a Sharples supercentrifuge. Centrifugation was between 40,000 and 46,000 rpm at about 2°C . The supernatant (Spn. I, p. 63) was collected and filtered under suction through a S1-Sterilizing filter #14 (obtained from Republic Seitz Filter Corp., Milldale, Conn.) at ambient temperature. The filtrate was then concentrated to approximately one-tenth of its original volume in a humidifier assembly at room temperature, with no observable loss in activity. Further

concentration to a smaller volume may be achieved using the flash evaporator arrangement at about 40°C and 10 mm pressure whenever necessary. Cells were collected and disrupted in a French press (American Instrument Co. Inc., Silver Springs, Maryland) at a pressure setting of 16K-22K. Disrupted cells were taken up in minimum amount of 20 mM sodium glycerophosphate buffer pH 6.4 and centrifuged to remove the broken cells.

Starting with 1 liter of the concentrated culture filtrate, the summary for the purification of BMA is depicted in the flow diagram (see p. 63). The step by step operations involved in the purification process are detailed below.

Heat treatment

The concentrated culture filtrate (1 liter) was centrifuged for 60 minutes at $16,000 \times g$ and 0°C to ensure the complete removal of cellular matter and debris and the supernatant (Spn. II) was adjusted to pH 6.4. It was then gently heated at 65°C in a water bath for fifteen minutes, followed by rapid cooling in crushed ice for 5 to 10 minutes. Denatured proteins were centrifuged off at $16,000 \times g$ for 1 hour at 0°C. The precipitate was discarded and the supernatant saved for subsequent treatments.

Ammonium sulphate precipitation

The supernatant obtained from the heat treatment step was brought to an ammonium sulphate saturation of $S = 0.55$ (~30°) by the addition (with slow stirring at 0°C) of finely milled $(\text{NH}_4)_2\text{SO}_4$ (enzyme grade preparation purchased from Mann Research Laboratories, Inc., New York, N. Y.). When all the salt had dissolved, the pH was adjusted to 6.4. After standing in the cold room for 18 hours, the solution was centrifuged for 60 minutes at

16,000 \times g. The precipitate (Ppt. I) was collected and stirred up in minimum amount of buffer. After further centrifugation, the supernatant was added to the original supernatant III and more solid ammonium sulphate added to $S = 0.80$ (~45%). After allowing to stand at 5°C for 6 hours, the precipitate (Ppt. II) was then separated by centrifugation at 16,000 \times g for 1 hour. The supernatant was discarded, and the precipitate was dissolved in minimum amount of 20 mM glycerophosphate buffer pH 6.4. Traces of insoluble matter was removed by filtration or further centrifugation. The solution obtained was then dialysed against periodic changes of 20 mM glycerophosphate buffer at 0°C for 48 hours. Any observable debris was filtered or removed by centrifugation.

Acetone fractionation

Ice-cold dry acetone was gradually added to the above solution to 66% (v/v). On allowing to stand in the cold for 60 minutes the precipitate formed was collected by centrifugation at 16,000 \times g for 1 hour. The supernatant was discarded and the acetone removed from the precipitate by applying gentle suction for 15 minutes at room temperature. The resultant brown cake was dissolved in minimum amount of buffer.

Gel filtration on Sephadex G-100

The protein solution obtained from the acetone precipitation was fractionated on a Sephadex G-100 column. A column (45 mm \times 450 mm) was packed using standard capacity Sephadex G-100. The gel was equilibrated with 20 mM glycerophosphate buffer pH 6.4 for 6 hours, and the packed column was washed at room temperature with 200 ml of the same buffer. Approximately 650 mg protein [as determined by Folin-Lowry method (77)]

in 10 ml solution was gently layered on the column. The column was then eluted with the same buffer, and 4-ml fractions were collected at a flow rate of 20 ml/hr. A typical elution pattern is shown on page 55. Active fractions were pooled and concentrated. The preparation may be stored at 0°C as a solution or lyophilized and stored in the freezer.

Ion exchange chromatography on DEAE-Sephadex A-50

The fractions from the Sephadex G-100 step containing *macerans* amylase activity were pooled and further fractionated by ion exchange chromatography. DEAE-Sephadex A-50 was swollen in 500 mM glycerophosphate buffer pH 6.4 for 3 hours in a boiling water bath. On cooling, the gel was equilibrated with periodic changes of 50 mM glycerophosphate buffer pH 6.4 for about 24 hours. A column (25 mm × 250 mm) was packed and equilibrated at room temperature by a continuous wash with 200 ml 50 mM buffer. About 20 mg-50mg protein was applied onto the column, and stepwise elution with 200 ml 50 mM glycerophosphate buffer and 200 ml 100 mM glycerophosphate buffer (both pH 6.4) was found preferable to a buffer gradient elution. The flow rate was 10 ml/hr and 3-ml fractions were collected. The typical elution profile is shown in Fig. 4. Active fractions were pooled and concentrated at 40°C under vacuum to one-fifth of the original volume. The concentrate was then dialysed against periodic changes of distilled water at 0°C for 48 hours, because high ionic strength was found to repress enzyme activity. The solution obtained after dialysis was stored in the cold for months as a solution without any significant loss of activity.

Properties of *B. macerans* AmylaseElectrophoretic pattern of BMA

Analytical polyacrylamide disc gel electrophoresis of purified BMA was conducted according to the 1965 Canalco formulation sheet. Electrophoretic runs were made at room temperature and pH values of 9.5 and 6.4 on both the enzyme preparation from Sephadex G-100 and the purified fraction from DEAE-Sephadex A-50. Satisfactory staining of the bands was achieved using trichloroacetic acid (TCA) and Coomassie brilliant blue as described by Chrambach *et al.* (78). The typical protocol was as follows: 1.5 ml of separating gel was carefully transferred into a 50 mm × 5 mm disc gel column. After the gel has polymerized (about 15 minutes), 0.4 ml of stacking gel was added and was photopolymerized using a three-bulb assembly of Sylvania 30W daylight fluorescent lamp with a large amount of blue light. The sample gel (0.2 ml) containing the required amount of protein was then added and also photopolymerized. Each gel segment was carefully layered with a drop of water before each photopolymerization step. This was found necessary to insure distinct gel interfaces. After adding 0.005% bromophenol blue per liter of buffer in the upper reservoir, electrophoresis was conducted using an ionic strength of 0.01 and a current of 5 ma per column at a potential between 280V-300V). At the completion of each run (usually about 150 minutes), the polyacrylamide cylinder was rapidly fixed in 12.5% TCA for at least 30 minutes. The gel was then stained with freshly made 0.05% Coomassie blue in 12.5% TCA for at least 1 hour. The gel was later transferred into 10% TCA and photographed. Under this condition of storage, the bands often remain distinct for at least two months.

For the location of active bands, electrophoretic runs were made at pH 6.4 followed by elution and activity testing at the same pH. The eluant buffer was 20 mM sodium glycerophosphate.

pH optimum and stability

BMA activity was determined at various pH values in the range 4-10.2. Two types of buffers were employed. Citrate buffer was used for pH values below 5.8 and glycerophosphate buffer for pH values above 5.8. The substrate was dissolved in the appropriate buffer at the desired pH, and the enzyme was suitably diluted with the same buffer. In view of its better quantitative adaptation the assay described by Thoma *et al.* (55) and modified by us was used in this study. It was necessary to avoid contribution by pH effect on glucamylase. Therefore, the assay was carried out in two stages. All reactions were conducted at 40°C.

The enzyme and solution A were incubated for 15 minutes at the pH of interest, followed by rapid boiling to stop the reaction. On cooling and adjustment of the pH of the digest to 4.5, 0.1 ml of solution B was added to initiate the second stage of the reaction. After 10 minutes incubation, the reaction was stopped by boiling and the reducing value determined by the alkaline potassium ferricyanide-cyanide method. In addition to this quantitative assay, the semiquantitative Tilden-Hudson microscopic slide assay was also tried.

In the pH stability studies, BMA alone was initially exposed to the pH of interest for 15 minutes. The pH was then rapidly adjusted to 6.4 on Beckman research pH meter fitted with a combination electrode. The pretreated enzyme was then added to solution A also at pH 6.4. After 10

minutes the reaction was stopped by boiling and on cooling the pH was adjusted to 4.5 and solution B was added. The reaction was again stopped after 10 minutes and the reducing value of the digest was determined using the standard procedure.

Temperature studies

The thermostability of BMA in both the presence and the absence of substrate was investigated. In order to isolate temperature effect due to BMA alone, the procedure followed in the pH studies was also adopted for these studies.

For the temperature optimum studies, 0.2 ml BMA was incubated with 0.8 ml solution A at the temperature of interest for various time intervals. After the reaction had been stopped by boiling and subsequent cooling, the pH was lowered to 4.5, 0.1 ml of solution B was added and the reaction allowed to proceed for 5 minutes at 40°C. The reaction was again stopped by boiling, and the reducing value was determined by the standard method.

In the investigations on heat stability, the enzyme alone was given a 10 minute heat shock at the appropriate temperature. After rapid cooling in crushed ice, the thermally treated enzyme was then used in the regular assay at 40°C.

Molecular weight determination

The molecular weight of BMA was estimated by the use of calibrated columns of Sephadex G-100 and Bio-Gel P-150. A calibration curve was prepared for either column using the following crystalline proteins of known molecular weight: Papain and ovalbumin (Calbiochem, Los Angeles,

California), β -lactoglobulin (Armour and Co., Chicago, Ill.), bovine serum albumin (Pentex Inc., Kankakee, Ill.) and *Aspergillus oryzae* α -amylase (purchased from Sankyo Co. Ltd., Tokyo, Japan).

A 100-ml burette was packed to the 5 ml mark with the properly swollen, degassed and equilibrated gel. 1 ml fractions were collected at a flow rate of 15 ml/hr (Sephadex G-100 column) and 10 ml/hr (BioGel P-150 column). 1 ml of 0.1% Blue Dextran 2000 (molecular weight 2,000,000) purchased from Sigma Chemical Company, St. Louis, Missouri, was employed in determining the void volume of the gel bed both before and after each molecular weight estimation. Protein peaks were identified by the Lowry method (77) on the Technicon Autoanalyzer, while BMA was identified by activity testing. Each protein was applied and eluted separately.

The column was calibrated by plotting the relative elution volume (V_e/V_0) of the standard proteins against their molecular weights using a semi-log paper (1 cycle \times 70 divisions). The best straight line was drawn to connect the data points. *Bacillus subtilis* α -amylase was used as marker proteins to check on the precision of the method.

Action of *B. macerans* Amylase

Reaction of BMA with various polysaccharides substrates

The action pattern of pure BMA was reexamined on a number of well characterized substrates such as amylose, amylopectin, glycogen, etc. Substrates were prepared in aqueous buffered solutions in which the concentrations ranged between 0.1% and 1.0% by weight. The enzyme was added so that there was usually about 0.10 Tilden-Hudson unit per milligram of substrate except in the cases of known poor substrates where up to 0.50 Tilden-Hudson unit per milligram of substrate were often employed. All reactions were performed at 40°C in a buffer system that contained 20 mM sodium glycerophosphate and 10 mM NaCl. The pH values varied between 6.2 and 6.5. The reaction was stopped by adding 0.1 ml 5M trichloroacetic acid to digest aliquots. This later proved to be a wise choice as BMA turned out to be able to regain activity on cooling if reaction was initially stopped by boiling.

Descending paper chromatography was extensively employed for the analysis of the products of the enzyme digests, except in some few cases where multiple ascending technique (79) was found more suitable. Water-nitromethane-95% ethanol (18:35:46 v/v) was used as the irrigating solvent for the multiple-ascent technique at 65°C. Usually up to three or four ascents were necessary for good resolution of compounds between G_1^1 and G_{12} , and clear separation of alpha, beta, gamma and delta dextrans. Descending chromatography was carried out in an airtight Chromatocab

1G_n designates a linear molecule of n glycosyl units linked by an α -1 \rightarrow 4 glycosidic bond.

(Model A125 Research Specialties Company, Berkeley, California) using 68% propanol-1 as the irrigating solvent. At 40°C and optimal atmospheric saturation of the chromatocab with solvent vapor, about 36 to 48 hours were often sufficient to resolve oligosaccharides down to G_{15} . In our hands this method gave superior resolution compared to multiple-ascent technique.

After proper irrigation, the dried chromatogram was rapidly dipped in a glucamylase solution (made by dissolving 1 gram Diazyme 160 in 80% cold acetone) according to the procedure of Kainuma and French (80). The wet chromatogram was air dried for about 5 minutes and then incubated in a moist chamber for 30 to 60 minutes at 40°C. This treatment enhances the intensity of each spot, since the glucamylase hydrolyzes the higher maltooligosaccharides to glucose. The chromatogram was dried again and then developed by the silver nitrate dip method as outlined by Robyt and French (69).

Detection of the cyclic dextrans was accomplished by subjecting duplicate chromatograms to methanolic I_2 -KI dip (10 ml of aqueous 0.2% I_2 , 2% KI; plus 200 ml of methanol) as described by Abdullah and French (81).

Radioautograms were prepared from digests of labeled substrates by exposing to Kodak no-screen medical X-ray film MS-54T in the dark for suitable length of time. Films were developed in Kodak liquid X-ray developer prepared in accordance with the manufacturer's instructions. The substrates used were starch, amylose, amylopectin, glycogen, β -amylase limit dextrans and the Schardinger dextrans (alpha, beta and gamma).

For starch, amylose, amylopectin and glycogen which are known to be

good substrates for BMA, a substrate concentration of 0.2% was buffered at pH 6.4. One tenth Tilden-Hudson unit of enzyme per milligram of substrate was added and the reaction was allowed to progress at 40°C. One hundred microliter aliquots of the digest were transferred at timed intervals to Whatman 3MM chromatography paper. After spotting the controls of malto-dextrin and Schardinger dextrin standards (twenty microliters of 1% solution), the chromatogram was subjected to descending paper chromatography at 40°C for 36 hours using 68% propanol-1 as irrigating solvent.

An identical procedure was followed for the investigations of the nature of the products formed by BMA action on β -limit dextrans and the Schardinger dextrans. However, 0.5 to 1% substrate concentration were used and two tenths Tilden-Hudson unit of enzyme was added per milligram of substrate.

Reaction of various BMA protein fractions with starch

The enzyme preparation obtained at each purification step, as well as the various protein fractions resolved on gel filtration chromatography and ion exchange chromatography were treated with 1% starch. The nature of the products formed were determined by paper chromatographic analysis. A typical digest consisted of 1.0 ml of 1% starch and 0.5 ml of the appropriate protein solution. The reaction mixture was incubated at 40°C for 3 hours (the time when starch is rendered achroic to iodine by BMA).

Two-dimensional paper chromatography of BMA digests

The action of BMA on α -dextrin, starch and amylose were surveyed in greater depth by two-dimensional chromatographic analysis as described by French *et al.* (82). This was done in order to specifically identify the

type of products obtained at the stage in the enzyme-substrate reaction where starch no longer gives a blue color to iodine. This point was found to be approximately 180 minutes for the concentration of BMA used in the starch reaction. Consequently, digests of amylose, α -dextrin, and starch withdrawn after 3 hours incubation with the enzyme were used for these studies. Instead of the multiple-ascent technique, the descending method was suitably adapted for this type of survey. The digest of BMA and the respective substrate was spotted along with maltodextrin and Schardinger dextrin standards. After irrigation at 40°C for 36 hours using 68% propanol-1, the reference channel was removed and developed in the usual way. The area containing the resolved oligosaccharides on the main chromatogram was given a thin spray of glucamylase-free β -amylase. It is important to exercise great caution when spraying the enzyme. The strip being sprayed should be damp but not running wet to minimize diffusion of the resolved spots. β -amylase solution was made up of 400 International units (IU) of enzyme in 10 ml of 100 mM pyridine-acetic acid buffer pH 4.8 (80). After further incubation at 40°C in a moist chamber for about 6 hours, the paper was dried, and new reference standards were spotted. The chromatogram was again irrigated along the direction 90° to the first direction for 33 hours. Upon drying, the chromatogram was given the regular treatment to identify the carbohydrate spots.

Mode of action of BMA

Reaction of BMA with uniformly labeled (UL) amylose Amylose

labeled uniformly in each of the glucosyl units was obtained by exhaustive

treatment of UL-¹⁴C-starch with pullulanase. Six milligrams (~0.1 mCi) of UL-¹⁴C-starch was dissolved in 2.9 ml of pH 6.5 buffer containing 20 mM sodium glycerophosphate and 10 mM NaCl. 0.1 ml (0.672 IU) of pullulanase was added, and the solution was incubated at 35°C for 15 hours. Reaction was stopped by boiling for 15 minutes and the digest upon concentrating to about 1 ml was carefully layered on a (15 mm × 400 mm) Sephadex G-25 column. The column was eluted with distilled water at a flow rate of 10 ml/hr. One milliliter fractions were collected and the radioactivity of the effluent was measured in a scintillation spectrometer. Six peaks were clearly resolvable, the first of which was pooled, concentrated, and rechromatographed on Sephadex G-100 column following identical elution conditions as above. Two separate peaks were obtained. Radioautograms of the two peaks showed that the first peak was homogeneous. Subsequent degradation of aliquots of this first peak by β-amylase gave about 95% conversion to UL-¹⁴C-maltose. This was then assumed to be pure UL-¹⁴C-amylose and was used without further purification.

A typical *B. macerans* amylase digest of UL-¹⁴C-amylose consisted of 1 ml (0.82 μCi) of labeled amylose, 20 mg amylose dissolved in 8.5 ml of buffer, and 0.5 ml (1.5 Tilden-Hudson units) of BMA. The reaction was allowed to continue at 40°C. At various time intervals, 0.8 ml aliquots were withdrawn into a test tube that contained 0.1 ml of 5M TCA. Fifty microliters of each aliquot was spotted on Whatman 3MM chromatography paper and the remaining portion was used for the iodine blue value determination on the Autoanalyser according to the procedure of Robyt and Demis (83). After proper irrigation of the chromatogram by the descending technique, the dried chromatogram was exposed to Kodak no-

screen medical X-ray film for 96 hours. On development, the spots corresponding to alpha, beta and gamma Schardinger dextrans, as well as the unreacted amylose at the origin were sectioned and counted on Packard scintillation spectrometer using the toluene scintillation fluid system.

Tritium labelling of BMA-amylose digest "Amylol" (amylose with the reducing end glucose unit reduced to a primary alcohol) was prepared by treating 50 ml of a solution containing 250 mg of amylose ($DP \approx 1800$) with $NaBH_4$ (25 mg $NaBH_4$ in 1 ml of 0.1N NaOH) for 3 hours at 65°C. On cooling 1 ml of 1N acetic acid was added and the mixture was stored at 40°C for not more than 5 days. Ten milliliter aliquots were passed over Sephadex G-25 column to remove the borate salts. Only one sharp carbohydrate peak was obtained. This was pooled and concentrated under vacuum at 50°C. The final amylool concentration was determined by the phenol-sulfuric acid method on the Technicon autoanalyzer in accordance with the procedure of Robyt and Bemis (83). Subsequent treatment of properly diluted aliquots of this solution with sodium borotritide showed no detectable uptake of tritium. It was therefore concluded that the reducing end glucosyl residue has been reduced to a primary alcohol.

Solutions containing varying concentrations of amylool between 0.1 mg/ml to 2 mg/ml were prepared by appropriate dilution of the stock amylool solution. Dilution was made with sodium glycerophosphate buffer pH 6.5. One tenth Tilden-Hudson unit of BMA per milligram of amylool was added and the solution was incubated at 40°C. 1.5 ml aliquots of the digest were withdrawn at specified time intervals and the reaction was stopped by boiling for 15 minutes. 0.5 ml of each aliquot was used for the iodine blue value determination.

The remaining 1 ml aliquots on cooling were each treated with a 20 to 25-fold molar excess of NaB^3H_4 in 0.1N NaOH at 65°C and pH 9 for 2 hours. Excess 1N acetic acid was added to destroy the unreacted borotritide. Care should be exercised at this step because of high tritium gas release.

The resultant solution was cooled for about 15 minutes and then evaporated to dryness under vacuum at 50°C. The residue was redissolved in 0.2 ml dimethyl sulfoxide (whenever found necessary) and diluted to 5 ml with distilled water. After thorough mixing for about 1 minute, the solution was again evaporated to dryness under vacuum. This process was repeated three times to ensure complete hydrogen exchange. The final residue was redissolved in 1 ml of distilled water. Five microliters of this was transferred into a counting vial and counted on the scintillation spectrometer with the dioxane scintillation fluid. Control blanks made up as follows were treated similarly.

1. Varying concentration of amylose 0.1 to 1 mg/ml.
2. Varying concentration of amylo1 0.1 to 2 mg/ml.
3. Equimolar mixture of Schardinger dextrans comprising of alpha, beta and gamma (5mM).
4. (^3H)-sodium borohydride (same concentration as used in the samples).

Gel filtration chromatography of (^3H)-sodium borohydride treated BMA-0.2% amylo1 digest

The digest aliquots of the (^3H)-sodium borohydride treated BMA-0.2% amylo1 at zero time, 30 minutes, 90 minutes and 180 minutes were separately layered on a 15 mm × 640 mm calibrated column of Sephadex G-50 fine. The column was eluted with distilled water at an average flow rate of 20 ml

per hour. Blue Dextran 2000 (molecular weight 2×10^6) was employed in determining the void volume. One milliliter fractions were collected, and monitored for total carbohydrate by the phenol-sulfuric acid method, and for radioactivity. The column was calibrated using a degraded amylose (DP 95) available in the laboratory. Each 1 ml fraction was collected and its total carbohydrate content and reducing value were determined. The mean DP was calculated from the ratio of the carbohydrate content to the reducing value.

RESULTS AND DISCUSSION

Production and purification

Progress in the biochemical investigations of *B. macerans* amylase has been severely handicapped by several factors. One nagging problem to investigators in this field has been the limitations imposed by the very long period of incubation required to get the organism to produce the enzyme. Several workers (44-47) have attempted to resolve this anomaly by various modifications of the culture medium. However, in the very few known cases where the period of incubation required to initiate enzyme production has been decreased, the yield of enzyme subsequently obtained has been very low (47). The production of amylases in a culture medium containing starch or glucose alone as the sole carbon source is noteworthy. Amylases in general have been found to be adaptive or inducible enzymes, and the macerans amylase has exhibited similar properties in this respect.

As a working hypothesis in this investigation, it was assumed that the lag between the period of active cell growth and the onset of enzyme production was probably due to the need for the organism to excrete carbohydrases that must first degrade the high molecular weight polysaccharide substrate into smaller fragments. These smaller fragments can then permeate the cell membrane and trigger the synthesis of BMA. Consequently, it was thought that the substitution of low molecular weight amylodextrins for starch as the source of carbon should decrease the lag period or eliminate it. This proved to be correct as shown in Table 1. The period required for *B. macerans* to start enzyme synthesis was decreased

Table 1. Production of *B. macerans* amylase by actively growing cultures of *B. macerans* as a function of the carbon source^a

Time (Days)	Activity in Tilden-Hudson units per ml of culture fluid			
	Soluble Starch	Maltodextrin (DP 44) or Maltrin 10 ^c	Maltrin 10 ^b	Maltodextrin (DP 44) ^b
0	-	-	-	-
2	-	0.5	0.5	1.2
3	-	0.5	1.0	1.8
4	0.2	1.0	1.5	2.0
7	0.2	1.0	1.0	2.5

^aPart of the data was obtained from the investigations of Katar and Robyt.

^bMore substrate added every 24 hours to keep the level of the carbon source at 0.5%.

^cMaltrin 10 is a commercial maltodextrin mixture obtained from Grain Processing Corporation, Muscatine, Iowa.

significantly. In their unpublished work, Katar and Robyt have looked into this problem. Employing paper chromatographic analysis, they observed rapid disappearance of the amyloextrin (DP \approx 44) from the culture medium between 20 and 30 hours after inoculation. Replacement of starch with glucose or any oligosaccharide in the range G₁ through G₄ did not produce any significant decrease in the lag period, and very often no enzyme was synthesized by the organism. The data obtained in the investigations reported here, clearly demonstrated the superiority of maltodextrins as

source of carbon supply over soluble starch. Rapid elaboration of BMA was observed usually within 2 to 4 days, compared to starch as the source of carbon which often takes 2 to 3 weeks. The effective use of the maltodextrins was greatly emphasized by the need to add more amyloextrin every 24 hours in order to keep the level constant. The exact substrate size requirement was not determined. However, comparison of the degree of enzymic activity in the medium when starch, maltrin 10 or maltodextrin (DP \approx 44) were separately used as carbon source showed that the last two substrates were used much more efficiently by the organism than starch. This may therefore explain the usual lag period observed by early investigators who used diced potato or rolled oats medium. It may well be that the initial response of the organism to the presence of high molecular weight polysaccharides is to synthesize carbohydrases (*e.g.* α -amylases) which fragment the polysaccharides into small sizes. The entry of these small molecular weight compounds then induces the organism to synthesize BMA. The best conditions for maximal cell growth and high enzyme yield were provided by buffering the medium with CaCO_3 at an initial pH of 6.8-7.0 and maintenance of this pH within the same range. It is suggested that the unusually high calcium salt precipitate that often attends the purification of BMA at each step, could be eliminated by feeding CaCO_3 into the medium through diffusion from a cellophane dialysis bag. Elaboration of *B. macerans* amylase was observed to be most rapid at 40°C.

Contrary to the observations of DePinto and Campbell (48) no intracellular production of BMA was detectable. However, a protein fraction that exhibited extensive cyclodextrinase activity was obtained intracellularly.

A second source of difficulty that has contributed largely to the complications in the study of BMA action, is the necessity of using an enzyme preparation free from other carbohydrases which might act on BMA substrates. This requirement was not met by early investigators. Preliminary experiments with small aliquots of the culture filtrate showed that the crude extract contains a large quantity of hydrolases along with a considerable amount of BMA. This conclusion was based on paper chromatographic analysis of starch digests of the culture filtrate. Relatively high amounts of reducing sugars were produced, and the loss of iodine-starch blue color was very rapid (usually within 10 minutes). As a first step in the purification of BMA, aliquots of the culture filtrate were passed over Sephadex G-25 column. Although this treatment removed the low molecular weight oligosaccharides, it was later omitted because there was no noticeable increase in specific activity. However, preheating of the culture concentrate at 65°C for 15 minutes was adopted since a significant increase in specific activity was achieved. A great deal of inert protein and hydrolytic enzymes were removed as precipitates by the heat treatment.

Initial precipitation studies with varying concentrations of $(\text{NH}_4)_2\text{SO}_4$ showed that a satisfactory fractionation of BMA could be quantitatively achieved by using salt concentrations ranging between 30% to 45%. A typical curve of specific activity versus concentration of salt as well as the specific activity precipitated at 45% $(\text{NH}_4)_2\text{SO}_4$ versus pH is depicted in Fig. 1. Better than 90% of the activity is precipitated at pH 6.4. This step offered the advantage of a large decrease in volume and an approximate nine-fold increase in specific activity.

Figure 1. Precipitation of BMA with $(\text{NH}_4)_2\text{SO}_4$

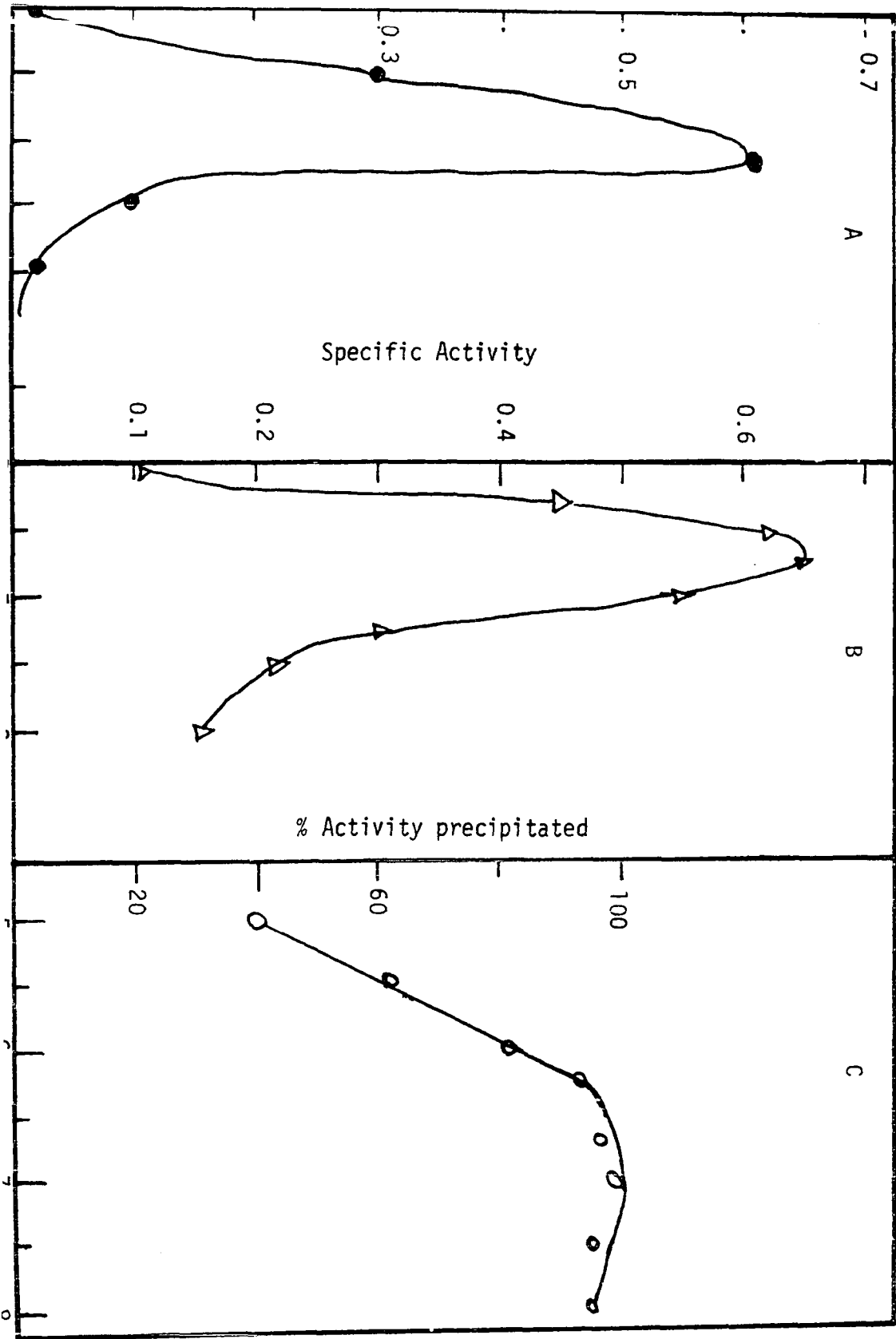
A - Precipitation at fixed pH = 6

B - Precipitation at 45% $(\text{NH}_4)_2\text{SO}_4$ and varying pH

C - Same as B

*Specific activity is based on Tilden-Hudson units

Specific Activity* (units/mg. protein).



Subsequent treatment of the enzyme preparation to an acetone concentration of 66% also led to further reduction in volume with no appreciable decrease in yield. A three-fold increase in specific activity with respect to the $(\text{NH}_4)_2\text{SO}_4$ step was obtained.

Chromatographic analysis of the enzyme preparation obtained after the acetone treatment revealed that a significant separation of the hydrolytic enzyme contaminant has not been achieved. The preparation was therefore subjected to gel filtration chromatography on the assumption that the two activities might be separable on the basis of molecular size differences. Figure 2 shows a typical elution profile of the acetone treated fraction on Sephadex G-100. Three distinct protein peaks were resolved. Peaks I and III showed no cyclic dextrin forming activity with 1% starch, and all the BMA activity was found in the protein peak II. The enzyme preparation at this point generally gives about 15-fold increase in specific activity. Examination of the active protein peak (fraction II) on polyacrylamide disc gel electrophoresis gave three distinct and two faint protein bands (1-5, Fig 3A). Only fraction 4 exhibited cyclic dextrin forming activity.

The electrophoretic behavior of fraction II protein on disc gel suggests that a successful separation of all contaminating proteins might also be possible using ion exchange chromatography on DEAE-Sephadex. This assumption was even reinforced on comparison of action pattern of the starch digests of the fraction II protein from Sephadex G-100 and that of the fraction 4 protein on disc gel electrophoresis (Fig. 3). It was observed that the products of the starch digest of the latter protein contained almost non-detectable reducing sugars, while the former protein digest still gives reducing sugars along with cyclic dextrans. The elution

Figure 2. Gel filtration of BMA on Sephadex G-100

———— $A_{280 \text{ nm}}$
----- Activity

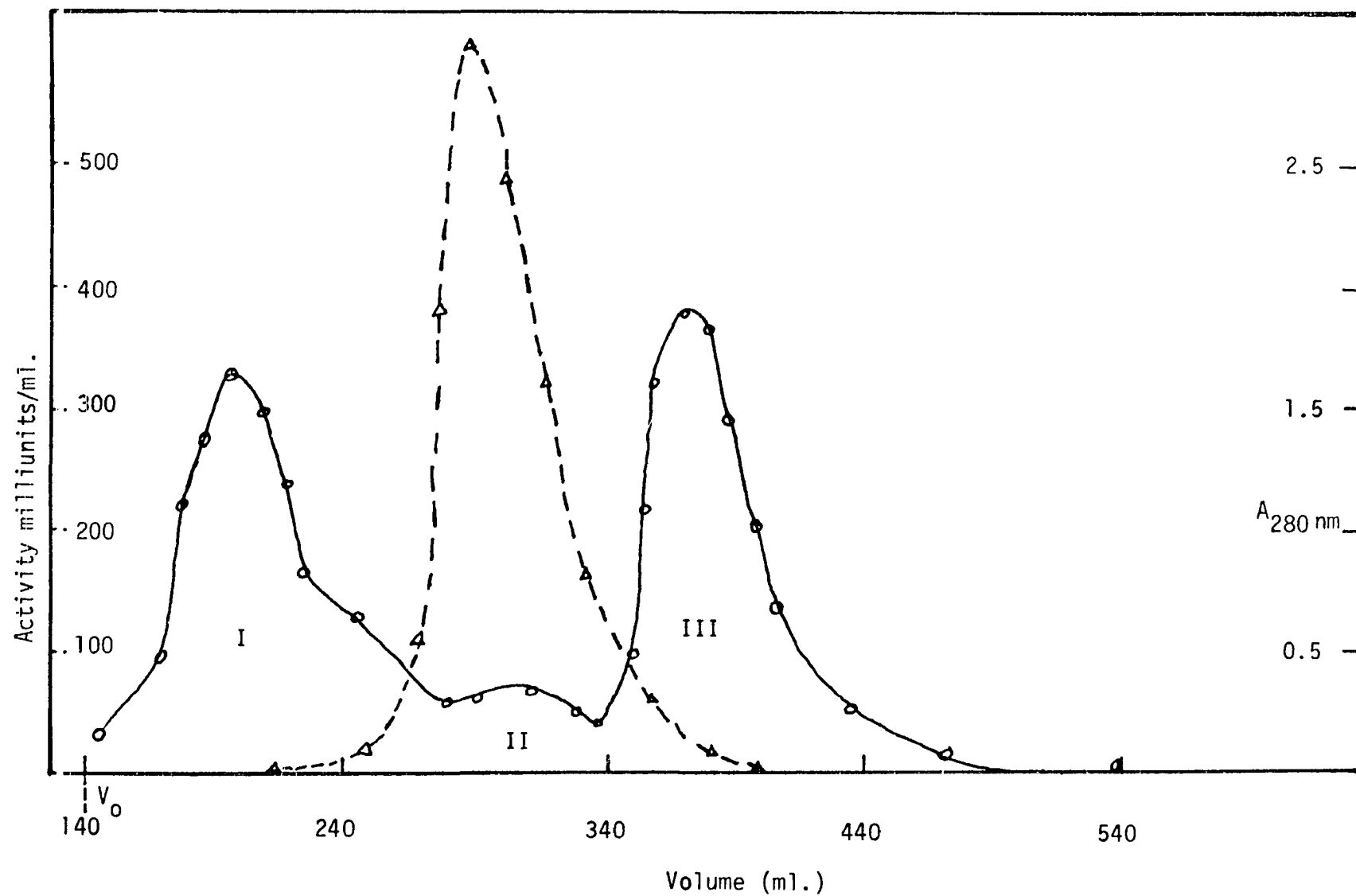
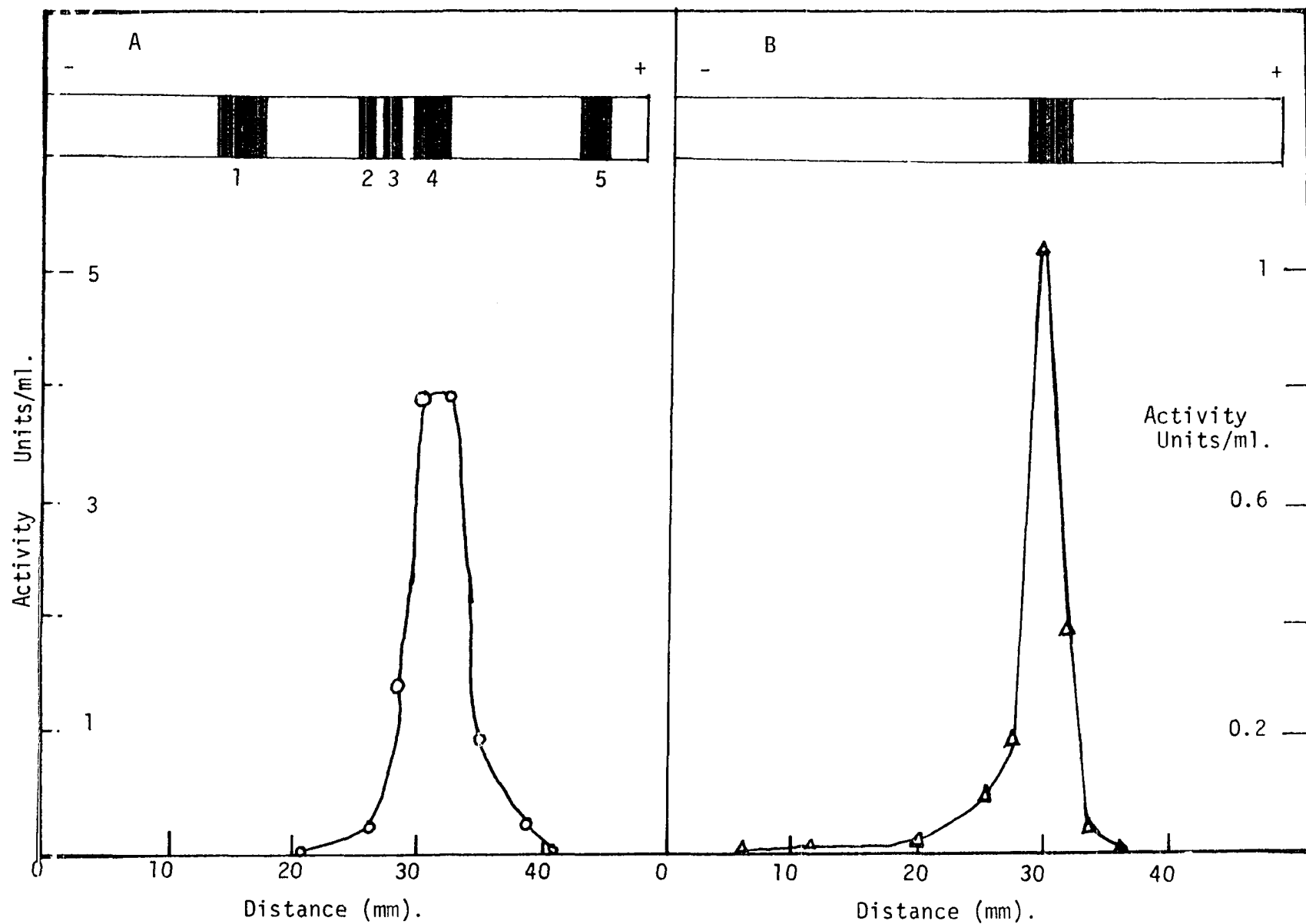


Figure 3. Polyacrylamide disc gel electrophoresis of BMA (pH 6.5 and 9.5)

A - Protein solution after the Sephadex G-100 step

B - Protein solution after the DEAE-Sephadex A-50 step

Running time = 150 minutes



pattern of fraction II protein on DEAE-Sephadex ion exchange column is shown in Fig. 4. Three protein peaks were clearly resolved. The peaks are named A, B, and C respectively in the order of their elution from the column. Only fraction B showed cyclodextrin-forming activity. The increase in specific activity is generally better than 300-fold with respect to the fraction obtained in the gel filtration step (Table 2, 3). It was observed that the degree of resolution on DEAE-Sephadex is dependent on the concentration of the contaminating proteins in fraction II. The height of the protein peaks is also a function of their quantity in the initial crude extract. The active protein obtained from this step (fraction B) exhibited one single band on disc gel electrophoresis over the concentration range of 30 $\mu\text{g/ml}$ to 300 $\mu\text{g/ml}$ of protein that was examined (Fig. 3B). Although paper chromatographic analysis of the pure enzyme obtained after the ion exchange chromatography showed no reducing sugars, certain preparations did. The only logical explanation for this appears to be in the proportion of contaminating proteins (2, 3, Fig. 3A) in fraction II protein. Since protein bands 2 and 3 migrated very close to the pure BMA band (band 4, Fig. 3A). on disc gel electrophoresis, it is likely that separation of these proteins on DEAE-Sephadex may not be complete. *A priori*, it would appear that successful separation of these two bands from BMA could be achieved on preparative polyacrylamide gel electrophoresis. However, by careful control of the flow rate on DEAE-Sephadex column, pure BMA that is free of these contaminants was obtained. In cases where the contaminants persisted, rechromatography on the same column eliminates them.

Table 4 depicts the summarized flow chart diagram of all the steps

Figure 4. Chromatography of BMA on DEAE-Sephadex A-50

————— $A_{280 \text{ nm}}$
----- BMA activity in $\frac{\text{mUnits}}{\text{ml}}$

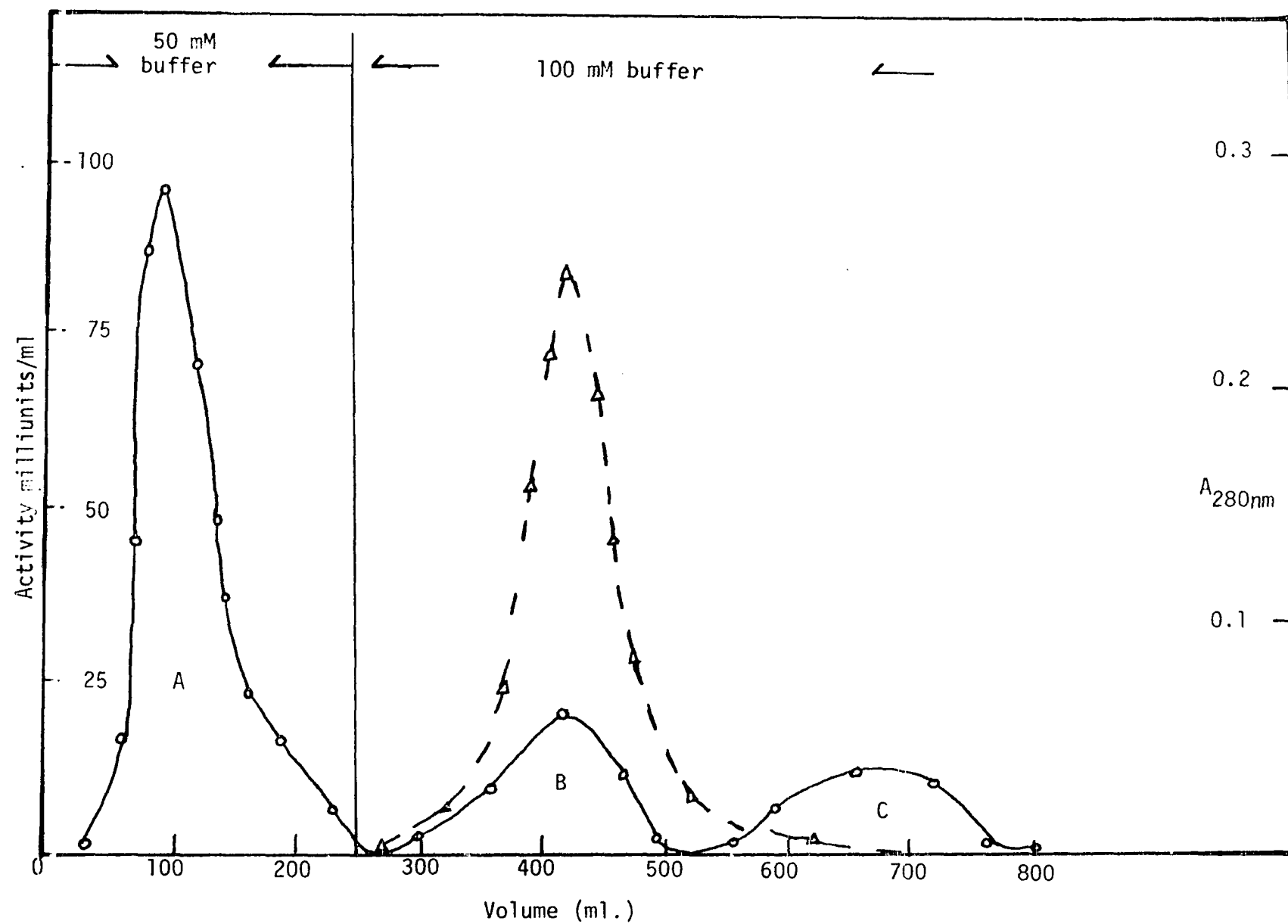


Table 2. Purification summary of *B. macerans* amylase

Procedure	Units per ml ^a	Total volume (ml)	Total units	% yield	Protein (mg/ml)	Total protein (mg)	$\frac{\epsilon_{280}}{\epsilon_{260}}$	Specific activity (units/mg)	Purification factor ^b
Culture supernatant	1	10,000	10,000	---	1.12	11,200	0.54	0.89	---
Culture concentrate	9	1,000	9,000	100	9.2	9,200	0.58	0.98	1
Heat treatment (15 min) 65°C	8.8	990	8,700	96.5	7.4	7,300	0.64	1.2	1.2
(NH ₄) ₂ SO ₄ S = 0.55 - 0.80 pH 6.4	40	132	5,280	59	4.4	580	1.06	9.1	9.3
Acetone precipitation 66% (v/v)	120	40	4,800	53	10.10	404	1.1	12	12.2
Sephadex G-100 pH 6.4	30	120	3,600	40	2.10	252	1.25	14.3	14.6
DEAE-Sephadex A-50 pH 6.4	5 ^c	54	270	30	0.015	0.81	1.54	333	333

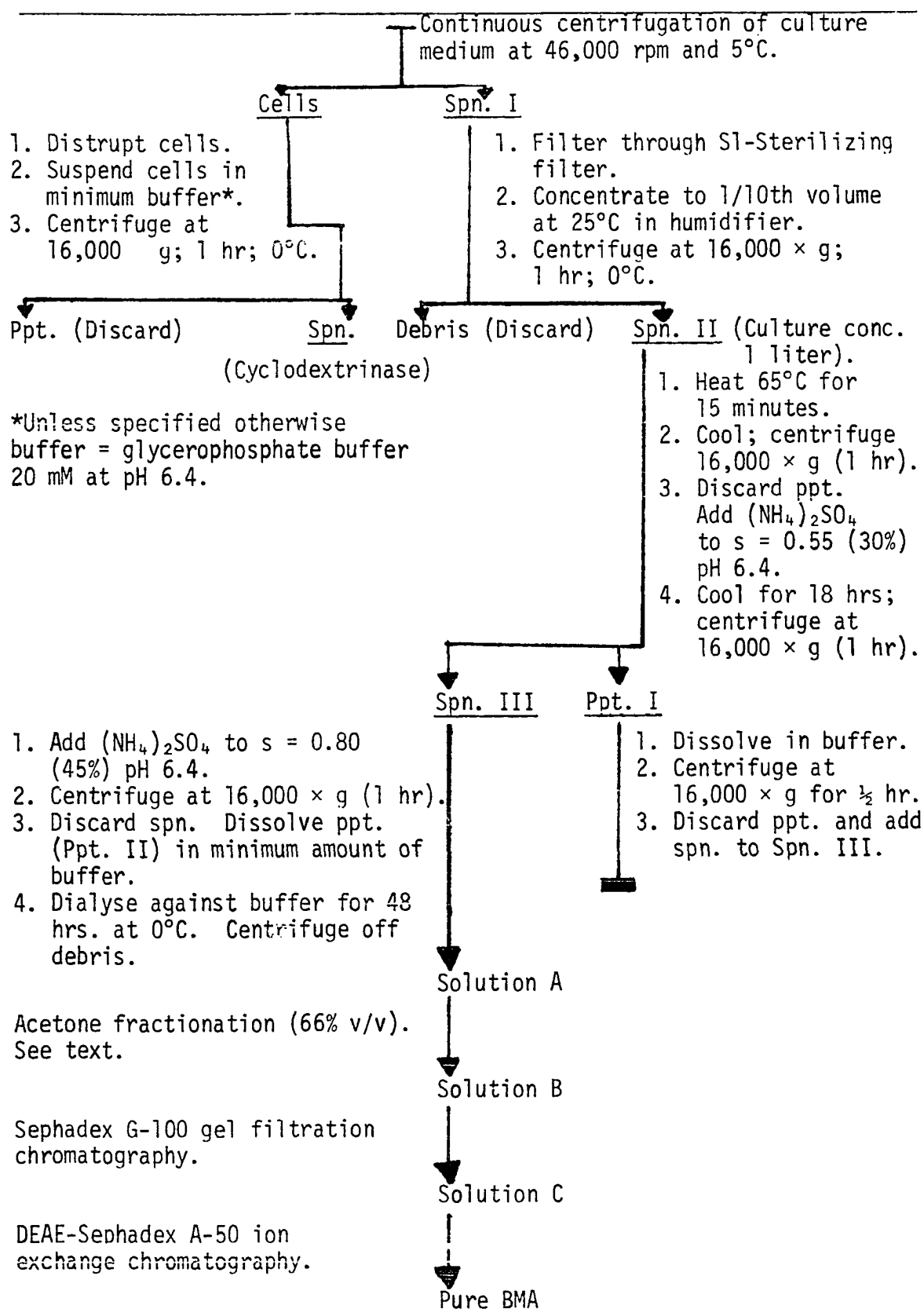
^aTilden-Hudson units.^bSpecific activity of purification step/specific activity of culture concentrate.^cBased on 12 ml aliquots of fraction obtained from Sephadex G-100.

Table 3. Purification summary of *B. macerans* amylase

Procedure	Units per ml ^a	Total volume (ml)	Total units	% yield	Protein (mg/ml)	Total protein (mg)	$\frac{E_{280}}{E_{260}}$	Specific activity (units/mg)	Purification factor ^b
Culture supernatant	0.09	10,000	900	---	1.12	11,200	0.54	0.08	---
Culture concentrate	0.85	1,000	850	100	9.2	9,200	0.58	0.093	1
Heat treatment (15 min) 65°C	0.84	990	830	97.5	7.4	7,300	0.64	0.12	1.3
(NH ₄) ₂ SO ₄ S = 0.55 - 0.80 pH 6.4	3	132	396	46.5	4.4	580	1.06	0.68	7.3
Acetone precipitation 66% (v/v)	9	40	360	42.3	10.1	404	1.1	0.90	9.7
Sephadex G-100 pH 6.4	2.5	120	300	35	2.1	252	1.25	1.2	13
DEAE-Sephadex A-50 pH 6.4	0.48 ^c	54	26	30.6	0.015	0.81	1.54	32	346

^aInternational units.^bSpecific activity of purification step/specific activity of culture concentrate.^cBased on 12 ml aliquots of fraction obtained from Sephadex G-100.

Table 4. Summarized purification diagram of BMA



involved in the purification process. A summary of the purification data is given in two parts in Table 2 and 3. Both methods of assaying that were used gave similar increase in specific activity. The removal of nucleic acids present in the culture filtrate (presumably due to autolysis) is indicated by the increasing $\epsilon_{280}/\epsilon_{260}$ values. The final product may be assumed to be virtually free of nucleic acids. Tests for the hydrolytic enzymes that produce reducing sugars in the culture filtrate preparations showed that the purified enzyme produces no reducing sugars. Pure BMA is free of carbohydrate as shown by the phenol-sulfuric acid test and it is stable in aqueous solution for at least several months at 5°C. Results clearly show that only a small fraction of the total protein in *B. macerans* culture filtrate is BMA. Although the amount varies between preparations an average value of about 0.2% was usually obtained.

Properties of BMA

Effect of pH Understanding the mode of action of BMA not only demands a knowledge of the possible structure of the substrate and products involved in the enzymic reaction, but also it is important to investigate the influence of environmental changes (*e.g.* pH and temperature) on the activity of the enzyme. Although by itself, pH studies of the enzyme will not furnish all the desirable information about the nature of the active groups on the catalytic site of the enzyme, certain inferences could be made from data obtained in such investigations.

B. macerans amylase has an optimum pH value between 6.2 and 6.5 (Fig. 5A). However, while the activity of BMA decreases on both sides of the optimum, the enzyme remains stable between pH 4 to 9.5 (Fig. 5B).

Figure 5. pH dependence of BMA activity

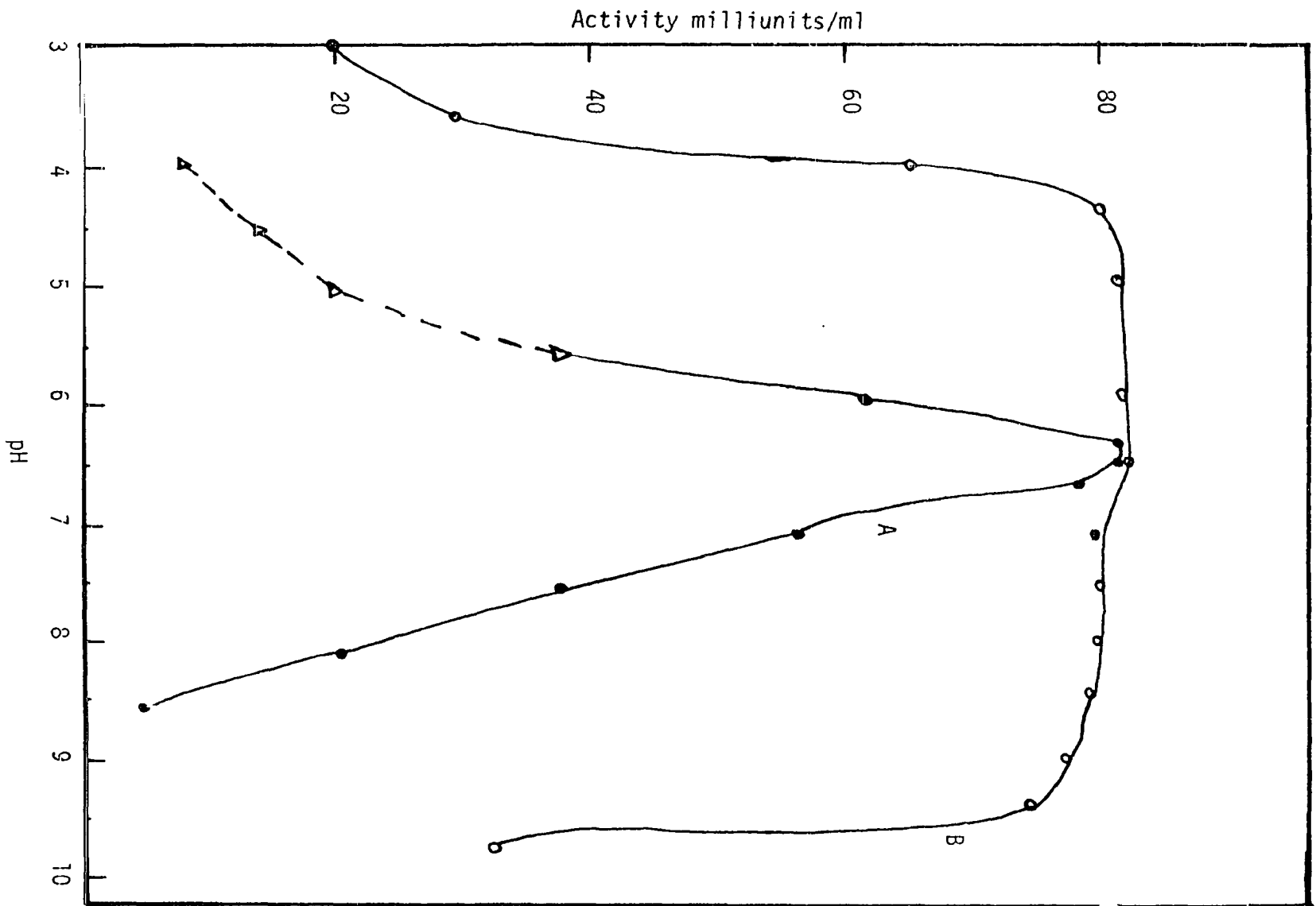
A - Effect of pH on activity

●—●—● Glycerophosphate buffer

Δ—Δ—Δ Citrate buffer

B - pH stability of BMA

For curve A the activity was tested at the pH values given;
for curve B the enzyme was exposed for 15 minutes to the pH
values given and the activity then tested at pH 6.4



Above pH 8.5 the enzyme had practically no activity, and it is essentially unstable below pH 4 and above pH 9.5. Since the catalytic activity of enzymes is often confined to a relatively small range of pH, it is definitely subject to pH control. The effect of pH on ionization of groups on the enzyme also influences the substrate binding as well as the molecular geometry of the protein. In general, the catalytic step in amylase action bears close resemblances to acid-base catalysis. Therefore the result shown in Fig. 5A may be reflections of the state of ionization of the enzyme, in which case the fall on either side of the optimum is due to a decreased saturation of the enzyme with substrate, or a true reversible effect on the velocity of products formation. From the bell-shaped nature of the pH activity curve and the pH values at which activity is about 50% of maximum, it would appear that certain reactive groups such as the β/γ -carboxyl groups of aspartic acid or glutamic acid and the imidazole group of histidine are participants in BMA catalysis. Schwimmer and Garibaldi (47) suggested that a tyrosyl group was involved in BMA catalysis. This speculation was based on stability studies of BMA in aqueous and iodine solutions at acid and alkaline pH values. The instability of BMA at high pH values (>9.5) where the tyrosyl residue is already in ionic form may be due to unfolding of the protein molecule resulting in conformational distortion of the enzyme.

Different values of the optimum pH have been reported for various BMA preparations from many laboratories. Tilden and Hudson (41) recorded a pH optimum of 5.6 to 6, Depinto and Campbell (52) obtained an optimum pH range of 6.1 to 6.2. The optimum pH of pure BMA is obviously above 6. However, it is not possible to conclude from the data reported here that

the pH dependence varies with the source of the enzyme, since the numerous different tests and substrates used prevent a strict comparison of the individual results.

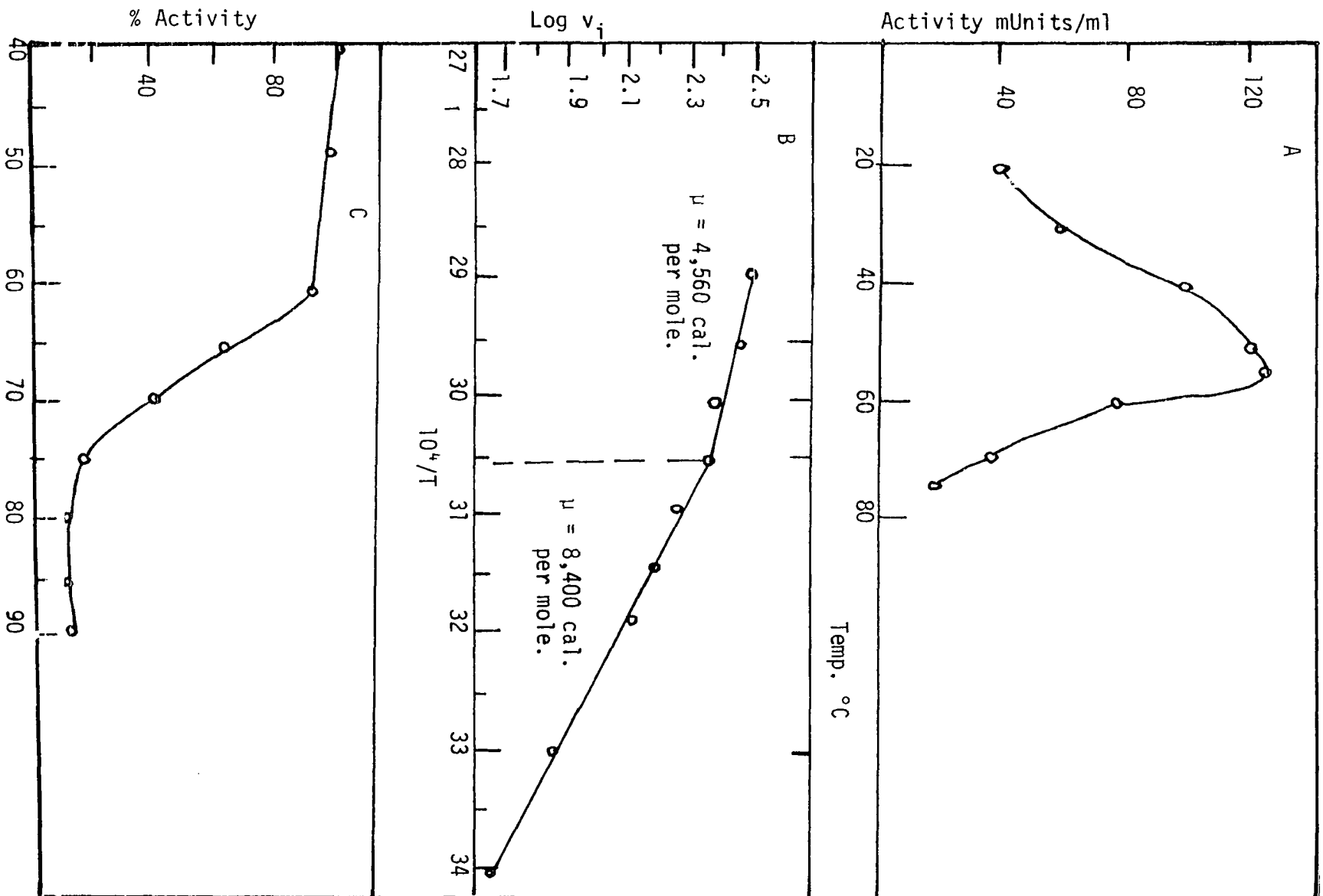
Effect of temperature The optimum temperature for BMA activity at pH 6.5 lies between 50°C and 55°C (Fig. 6A). The usual procedure was followed in studying the effect of heat on BMA by plotting $\log k = (\mu/2.303R)(1/T)$, where k is the reaction velocity, μ the activation energy, R the gas constant, and T the absolute temperature. The Arrhenius plot of velocity of reaction versus reciprocal of absolute temperature indicates gradual activation in the range from 15°C to 55°C (Fig. 6B). An inflection near 55°C corresponding to a change in the activation energy of the reaction was observed. This in similar cases, has been attributed by Dixon and Webb (84) and Massey *et al.* (85) to a transition of the enzyme between active conformations. A μ value of 8.4 kilocalories per mole was calculated from the slope of the line between 20°C and 55°C, whereas between 55°C and 75°C a μ value of 4.56 kcal per mole was obtained. The μ value of 8.4 kcal contrasts sharply with the value of 12.25 kcal per mole reported by DePinto and Campbell (52). The difference probably reflects differences in the state of purity of the enzyme preparations, or it could be due to different methods of assaying used. However, compared to the typical nonenzymic processes [*e.g.* acid hydrolysis of starch at 20°C, $\mu \approx 30$ kcal per mole (86)] the rapid lowering of activation energy is very significant. BMA is about twice as active at 55°C as at 40°C. The enzyme is slowly inactivated at temperatures above 55°C. The results of the heat stability investigations (Fig. 6C) clearly indicate that BMA is completely stable up to about 65°C, but it is reversibly denatured at

Figure 6. Temperature dependence of BMA activity in 20 mM sodium glycerophosphate buffer (pH 6.4)

A - Effect of temperature on BMA activity

B - Arrhenius plot of data from temperature effects on BMA activity

C - Heat stability of BMA

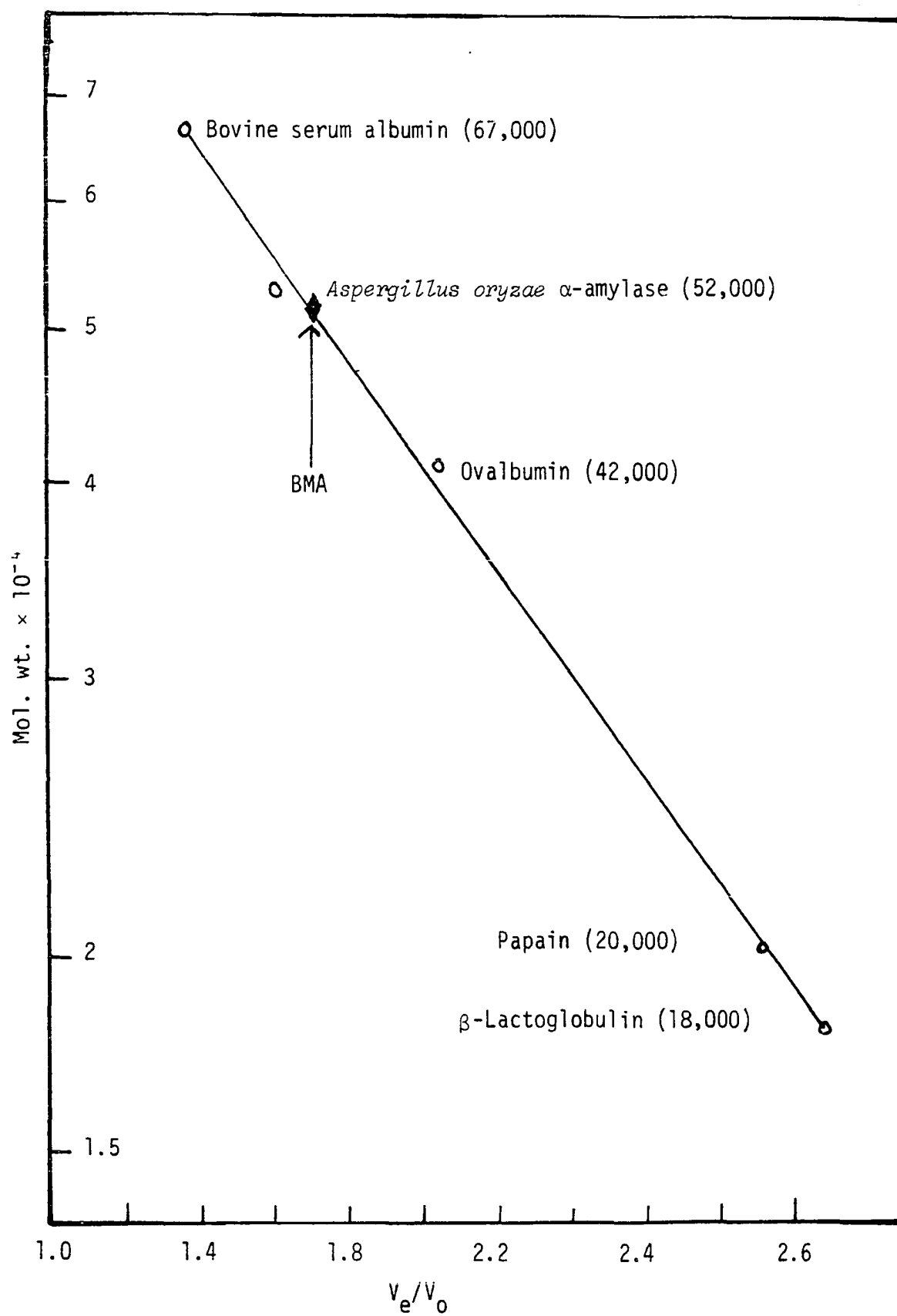


higher temperatures. As indicated earlier, BMA is capable of regaining about 50% of its activity after heating for 15 minutes at 100°C and cooling for about 24 hours. This relatively high thermostability in both the presence and absence of substrate appears to be characteristic of bacterial amylases.

Molecular weight determination The molecular weight of *B. macerans* amylase was determined from experiments employing gel chromatography as described by Whitaker (87). Under identical elution conditions as the standard proteins used in calibrating the gel column, BMA gave a value of V_e/V_o of 1.7 on Bio-Gel P-150 (Fig. 7). This corresponds to a molecular weight of $50,000 \pm 2500$. However, approximately half this value (27,500) was obtained when a Sephadex G-100 column was used for molecular weight estimation. The use of Sephadex (a cross-linked dextran) for determining the molecular weights of carbohydrates and polyglycanases is questionable because an interaction between the gel and the enzyme may occur, leading to retardation and low estimations of molecular weight. This appears to be the case with BMA elution on Sephadex G-100. This assumption was corroborated by the elution behaviour of *Bacillus subtilis* α -amylase. This enzyme in native form exists as a dimer (molecular weight 96,000). Using dilute concentrations of *B. subtilis* α -amylase (500 $\mu\text{g/ml}$ to 800 $\mu\text{g/ml}$) molecular weight values of 94,000 (dimer) and 49,000 (monomer) were obtained on Bio-Gel column, while the Sephadex column gave molecular weight values of 64,000, and 34,000 respectively. In view of this anomaly, the molecular weight data obtained for BMA on Bio-Gel column was therefore regarded as more correct.

Several workers have speculated on the molecular size of BMA, and

Figure 7. Determination of molecular weight of BMA by gel filtration chromatography on Bio-Gel P-150



different values have been reported in the literature. Schwimmer and Garibaldi (47) recorded a minimum molecular weight of 54,000; Schwimmer (56) reported 100,000; and DePinto and Campbell (52) suggested a minimum molecular weight of 140,000. Since no evidence of subunit structure of this enzyme was found in the investigations reported here, it is assumed that pure BMA in the native state probably has a molecular weight close to 50,000.

Action pattern of BMA

Reaction of BMA with polysaccharide substrates The nature and sequence of products formed by the action of BMA on a variety of substrates is depicted in Figs. 8 to 11. Products were detected by a combination of the silver nitrate dip method and the methanolic I_2 - KI technique described earlier. No chromatographically resolvable reducing sugars are produced from starch, glycogen, amylose and amylopectin (Fig. 8A - 8D). Duplicate chromatograms were treated with the methanolic I_2 - KI solution to detect the cyclic dextrans. By comparing the R_F of the samples with those of the standards, the first top three spots (Fig. 8A - 8D) are identified as α -, β -, and γ -cyclic dextrans. Detection of the other spots below γ -cyclic dextrin was accomplished by two-dimensional chromatographic survey of the various digests interspersed with β -amylase treatment on paper. Results of a typical survey of starch, and amylose are shown in Figs. 9 and 10. Essentially the same chromatographic pattern as shown for starch (Fig. 9) was obtained for amylopectin and glycogen. It is seen that all the spots except for the origin are resistant to β -amylase attack. It is therefore concluded that the initial primary

Figure 8. Products of the action of BMA on various polysaccharide substrates

R and R₁ are maltodextrin and cyclic dextrin reference compounds respectively. Samples of 100 μ l were spotted at various times. Reaction time was 180 minutes except otherwise indicated.

A - Starch

B - Amylose

C - Amylopectin

D - Glycogen

E - Waxy maize starch β -limit dextrin (74 hours)

F - Shell-fish glycogen β -limit dextrin

G - Fraction 4 protein + 1% starch, see Fig. 3A

H - Fraction II protein + 1% starch, see Fig. 2

I - α -Schardinger dextrin

J - β -Schardinger dextrin (74 hours)

K - γ -Schardinger dextrin (72 hours)

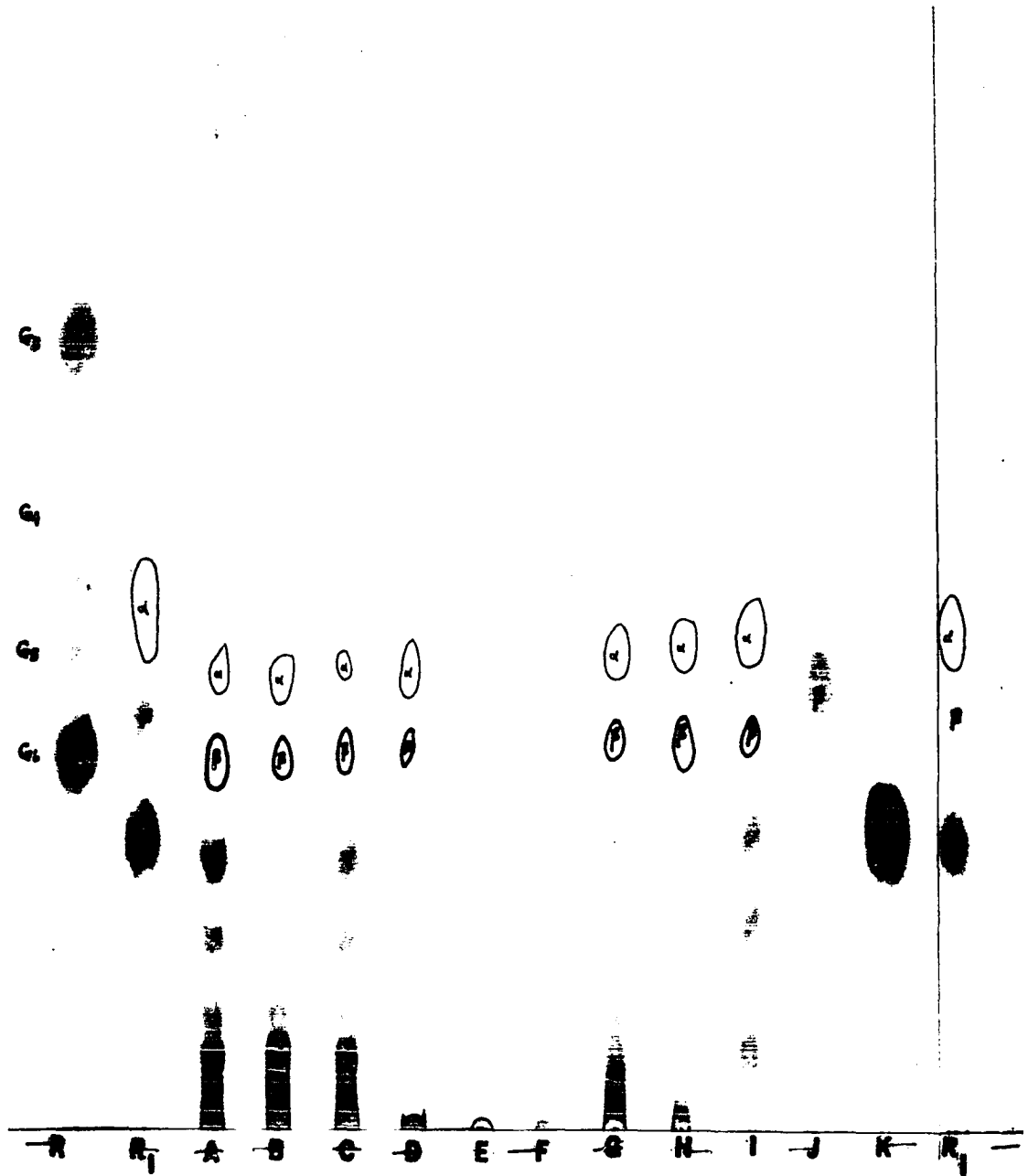


Figure 9. Two-dimensional chromatogram showing action of β -amylase on BMA starch digest

R_1 and R are cyclic dextrin and maltodextrin reference compounds respectively. S indicates the point of application of the sample.

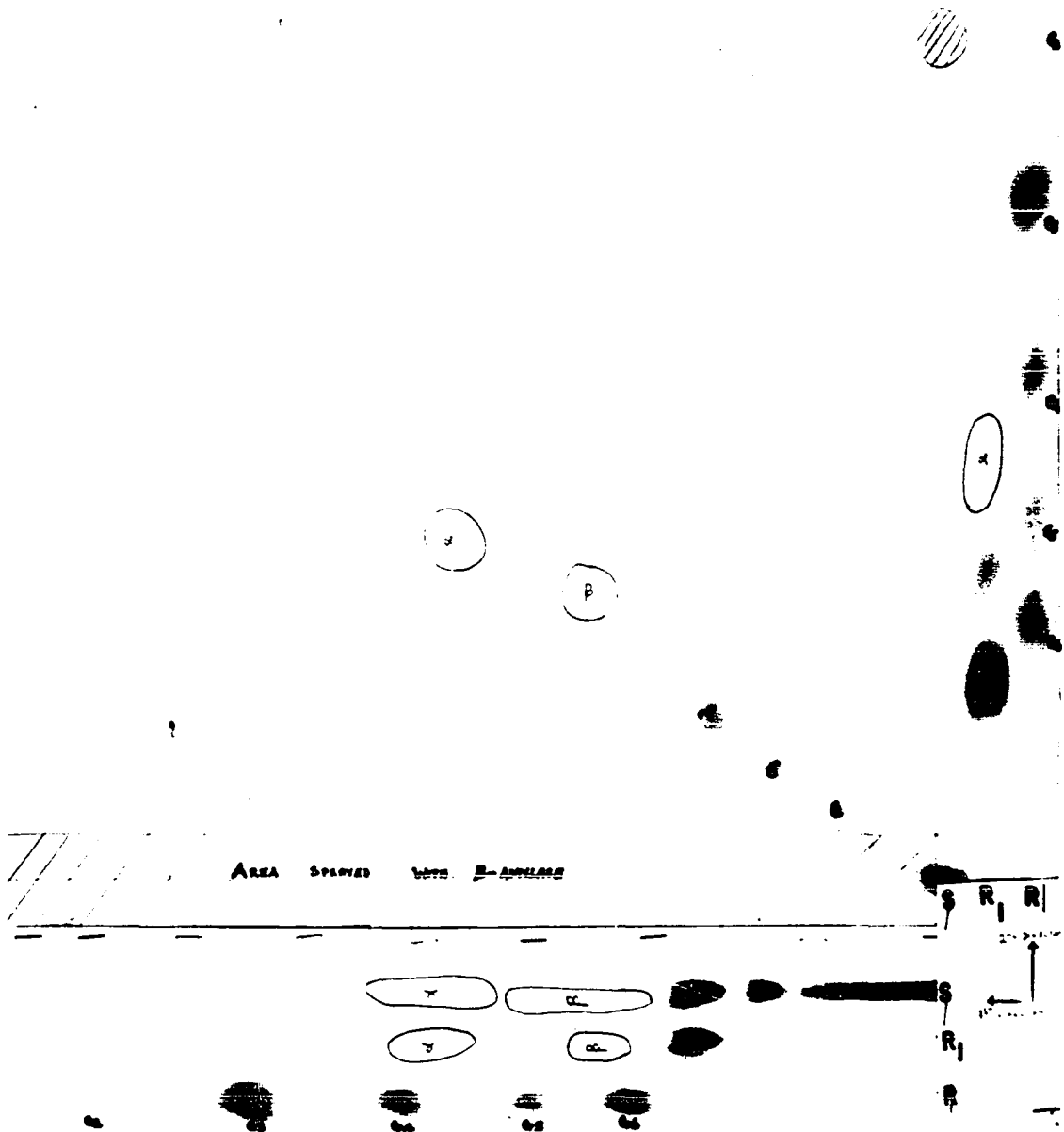


Figure 10. Two-dimensional chromatogram showing action of β -amylase on BMA amylose digest

R_1 and R are the cyclic dextrin and maltodextrin reference compounds respectively. S indicates the point of application of the sample.

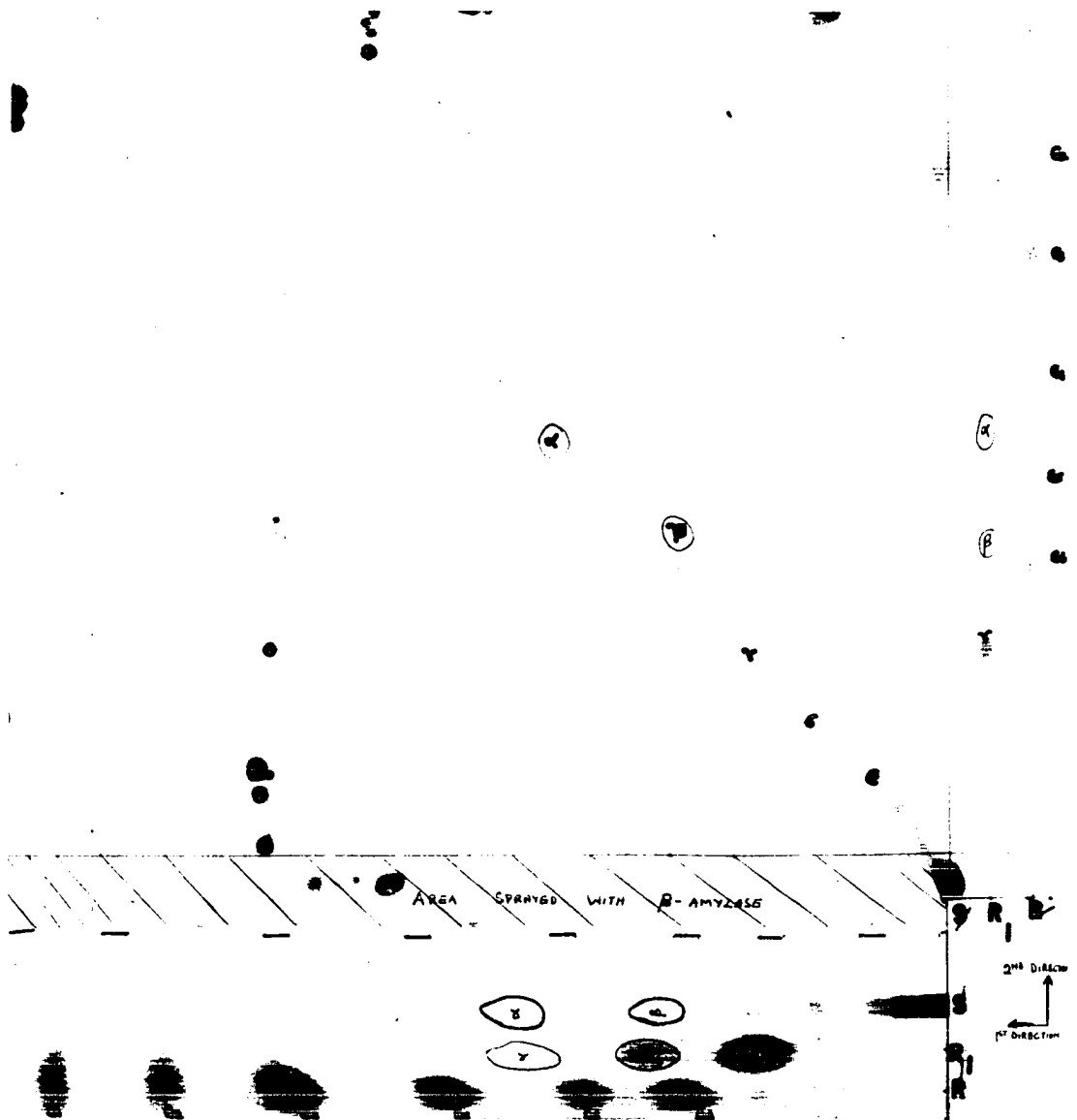
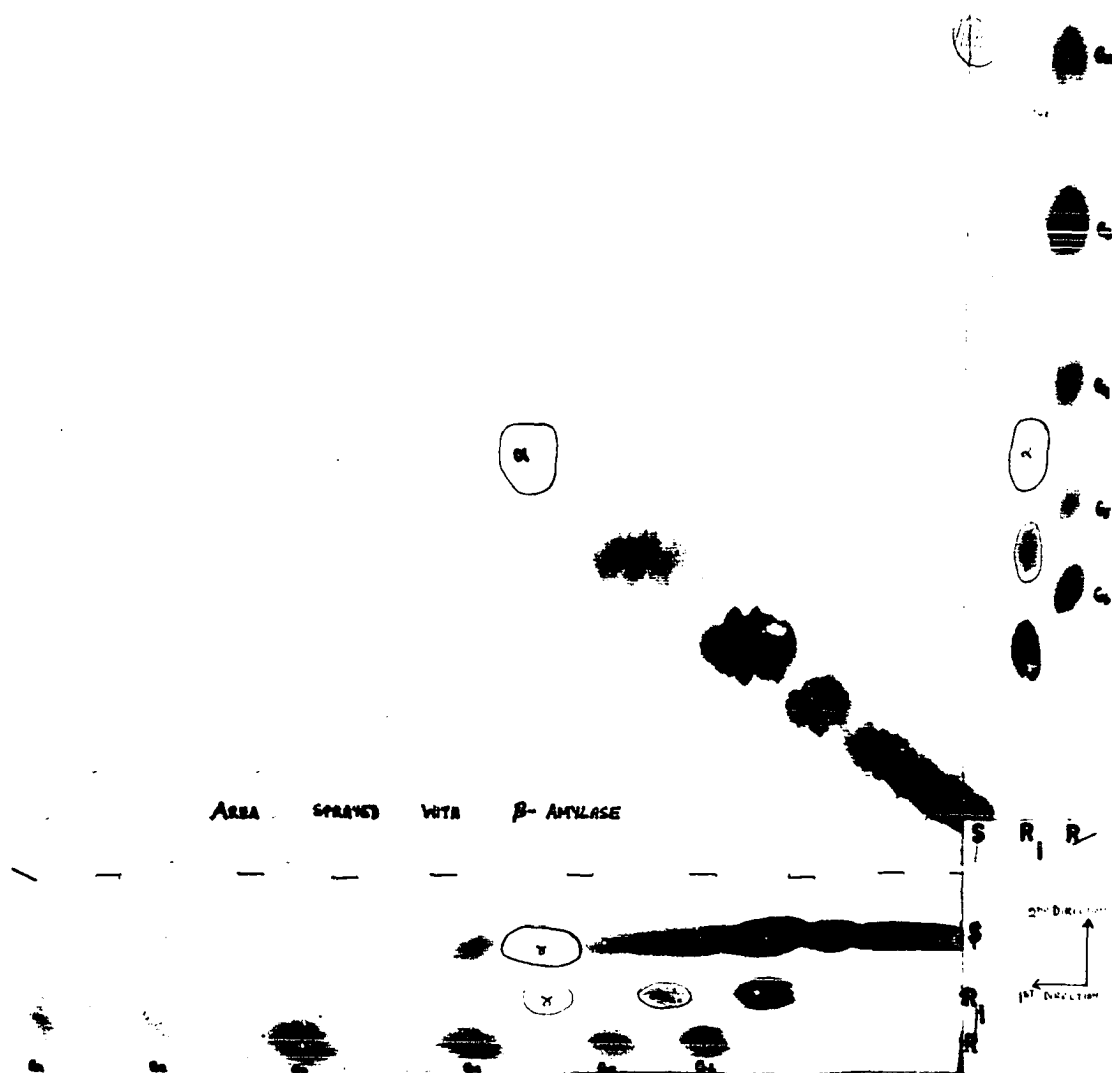


Figure 11. Two-dimensional chromatogram showing action of β -amylase on BMA α -dextrin digest

R_1 and R are the cyclic dextrin and maltodextrin reference compounds respectively. S indicates the point of application of the sample.



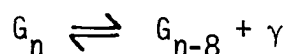
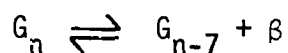
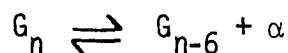
products of BMA action on starch, amylose, amylopectin and glycogen are exclusively the homologous series of cyclic dextrans. The production of maltose (G_2) (after the β -amylase treatment) at the origin (Figs. 9 and 10) is due to either unreacted substrates or unresolvable amylodextrins.

Both waxy maize starch-, and glycogen- β -limit dextrans (Fig 8E and 8F) as well as β - and γ -Schardinger dextrans (Fig. 8J and 8K) are very resistant to BMA attack. After incubation of these substrates with the enzyme for as long as 300 hours, there was no significant detectable products formed. However, the α -Schardinger dextrin (Fig. 8I) is shown to react readily with BMA to give a whole array of products. By means of two dimensional chromatographic analysis the products of BMA digest of α -dextrin are identified as mixtures of linear maltodextrins and cyclic dextrans (Fig. 11). In interpreting the two-dimensional chromatogram in Fig. 11, it must be borne in mind that all compounds that have not reacted with the sprayed β -amylase during the reaction on paper fall along an imaginary diagonal line drawn from the origin to the left-hand corner of the chromatogram. Compounds above and below this line are reaction products of β -amylase action. From Fig. 11 it is therefore concluded that BMA initially converts α -dextrin into β -, γ -, δ - (and possibly higher cyclic homologues) Schardinger dextrans, and maltooligosaccharides from G_2 upwards. Production of G_2 at the origin definitely indicates the presence of non-chromatographically resolvable maltodextrins resulting from homologizing or coupling reactions of BMA.

The results from Fig. 8, 9, and 10 clearly demonstrate that BMA does not form chromatographically detectable reducing sugars in the initial stages of its action on high molecular weight polysaccharides such as

starch, amylopectin, glycogen etc. This observation contrasts sharply with the results of DePinto and Campbell (53), who obtained significant production of reducing sugars ($G_1 - G_7$), when their BMA preparation was allowed to act on starch, amylopectin and glycogen. The results from the two-dimensional survey of starch and amylose digests (Figs. 9 and 10) and the radioautogram of UL- ^{14}C -amylose digest (Fig. 12) clearly substantiates the proposal originally advanced by French (2), that the source of reducing sugars in BMA digests is due to the presence of traces of hydrolytic enzymes in BMA preparations.

The complete resistance of β - and γ -Schardinger dextrans to BMA attack is not unexpected. Norberg and French (67) have established that BMA reaction is reversible according to the following equations,

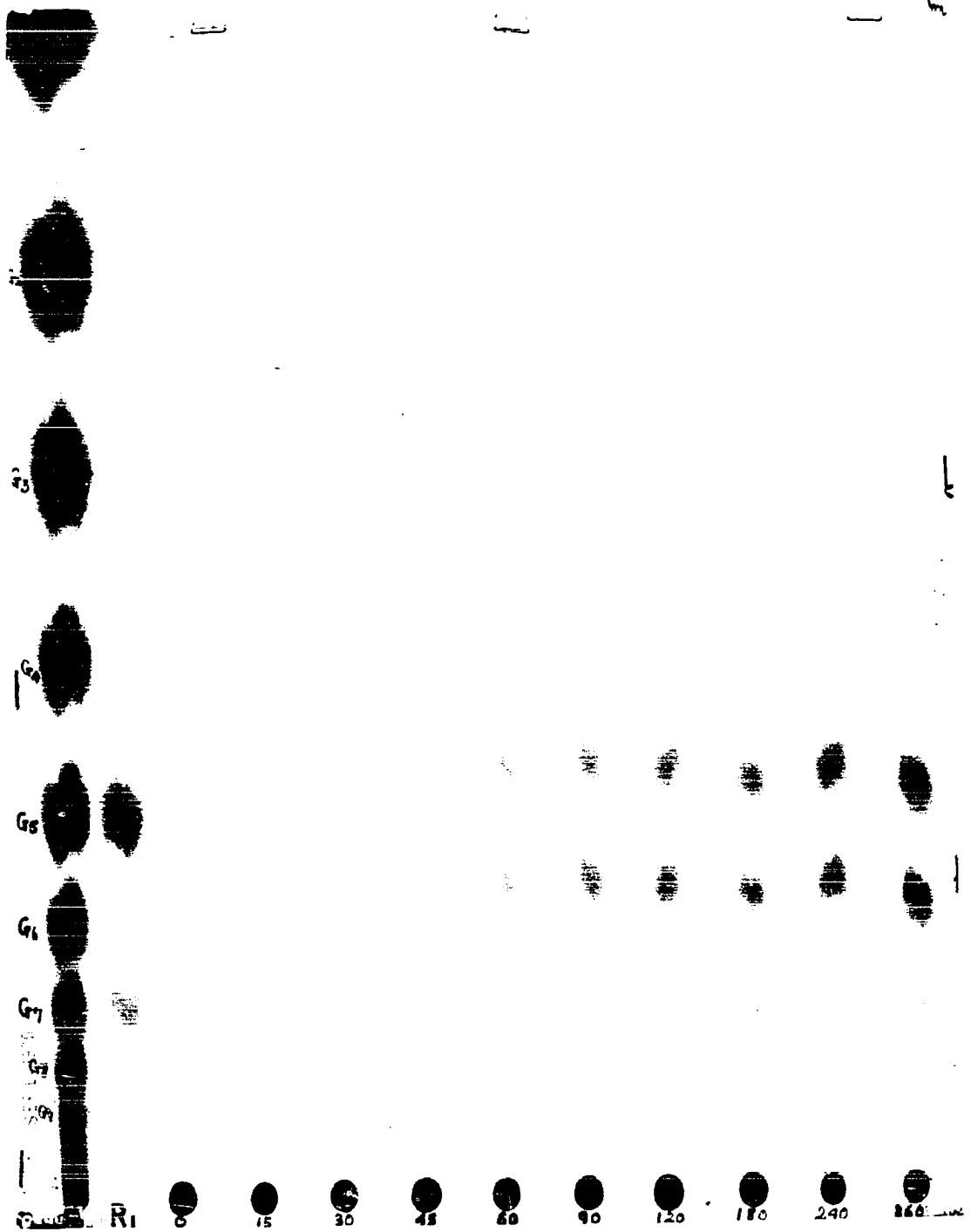


etc. where G_n is a polymer of n glycosyl units linked through an α -1 \rightarrow 4 glucosidic bond. From the above equations one ordinarily would not expect the reverse reaction to take place without an acceptor molecule present. Failure to observe any reaction of BMA with both β - and γ -cyclic dextrans alone as substrate is therefore understandable. However, the susceptibility of α -cyclic dextrin to BMA attack is anomalous. One possibility might be that the conversion of α -dextrin by the enzyme into the products indicated in Fig. 11 is essentially via coupling reaction initiated by undetectable traces of oligosaccharide contaminants such as maltopentaose which migrates very close to α -dextrin on paper chromatograms. A second

Figure 12. Time course radioautogram of the action of BMA on UL- ^{14}C -amylose

R indicates maltodextrin reference compound ($\text{G}_1 - \text{G}_{12}$).

R_1 is a mixture of G_5 and G_7 . The arabic numerals indicate digest time in minutes.



possibility may be the contaminating branched cyclic dextrans which also may not be detectable on paper chromatograms. In this regard French *et al.* (88) have reported that BMA starch digests in addition to the regular alpha, beta, and gamma dextrans, often contain branched cyclic products usually of the higher homologues. The α -dextrin used in this study showed no detectable contaminant as judged by the homogeneity of the α -dextrin spot on a paper chromatogram at zero time of reaction. However, the presence of chromatographically undetectable amounts of maltodextrans may trigger coupling reactions in which case the formation of higher cyclic dextrans observed in Fig. 11 is probably due to the coupling reaction with maltodextrans in the α -dextrin preparation. A similar result would be obtained if the contaminants were a branched cyclic dextrin. The prerequisite for coupling reactions to occur is the presence of a free C hydroxyl group. Both maltopentaose and any branched cyclic dextrin have a free C₄ hydroxyl group, and are, therefore, potential acceptor molecules in the coupling reaction. By preliminary treatment of α -dextrin with pullulanase (which hydrolyses α -1 \rightarrow 6 linkages in the branched cyclic compound) and β -amylase (which degrades linear oligosaccharides to maltose and maltotriose), these likely contaminants could be removed from the α -dextrin by chromatographic purification.

The resistance of β -limit dextrans (Fig. 8E and 8F) to BMA attack is in agreement with the expected mechanism of action of BMA. If BMA attacks its substrate by an exo-mechanism like β -amylase, it would be expected that the enzyme may be unable to bypass the branching points in non-linear substrates such as β -limit dextrans. The question, however, remains to be answered as to how the branched cyclic dextrans are formed

if it is indeed true that BMA cannot bypass branch points. On the basis of the initial difficulties experienced with certain preparations of β -limit dextrans, that are readily converted into cyclic products by BMA, it seems that a minimum number of glucose units must be left on the B chain of a branched substrate in order for BMA to form an exo- or an endo-cyclic dextrin. Below this minimum the substrate becomes resistant to attack, and the enzyme is prevented from incorporating the branches into products. Recently, Abdullah and French (81) provided evidence for the existence of a series of exo-branched cyclic molecules based on α -dextrin. They found chromatographic evidence for side chains of 2-7 glucose units. The data obtained here suggests that pure β -limit dextrans with primarily maltosyl or maltotriosyl A chains are resistant to BMA attack.

Reaction of various BMA protein fractions with starch It has been reported by several investigators (45, 32) that *B. macerans* secretes in addition to BMA, other enzymes capable of degrading starch. In an attempt to determine the effect of these contaminating proteins of BMA crude preparations on starch, all the various protein fractions isolated at each purification step were tested chromatographically using 1% starch as substrate. As shown in Fig. 13, the action patterns of the various proteins on starch are very similar. Except for the pure enzyme (#5 and #7 in Fig. 13) the other protein fractions gave significant reducing sugars in addition (in certain cases) to cyclic dextrans. Other than BMA, the different proteins obtained after gel filtration and ion exchange chromatography were not identified, but on the basis of their elution behavior they appear to have different physical properties. The hydrolytic contaminant (*i.e.*, the protein fraction that is responsible for the

Figure 13. Products of the action of the different proteins obtained in the purification of BMA on 1% starch

1 and 13 are maltodextrin reference compounds

12 is the cyclic dextrin standards

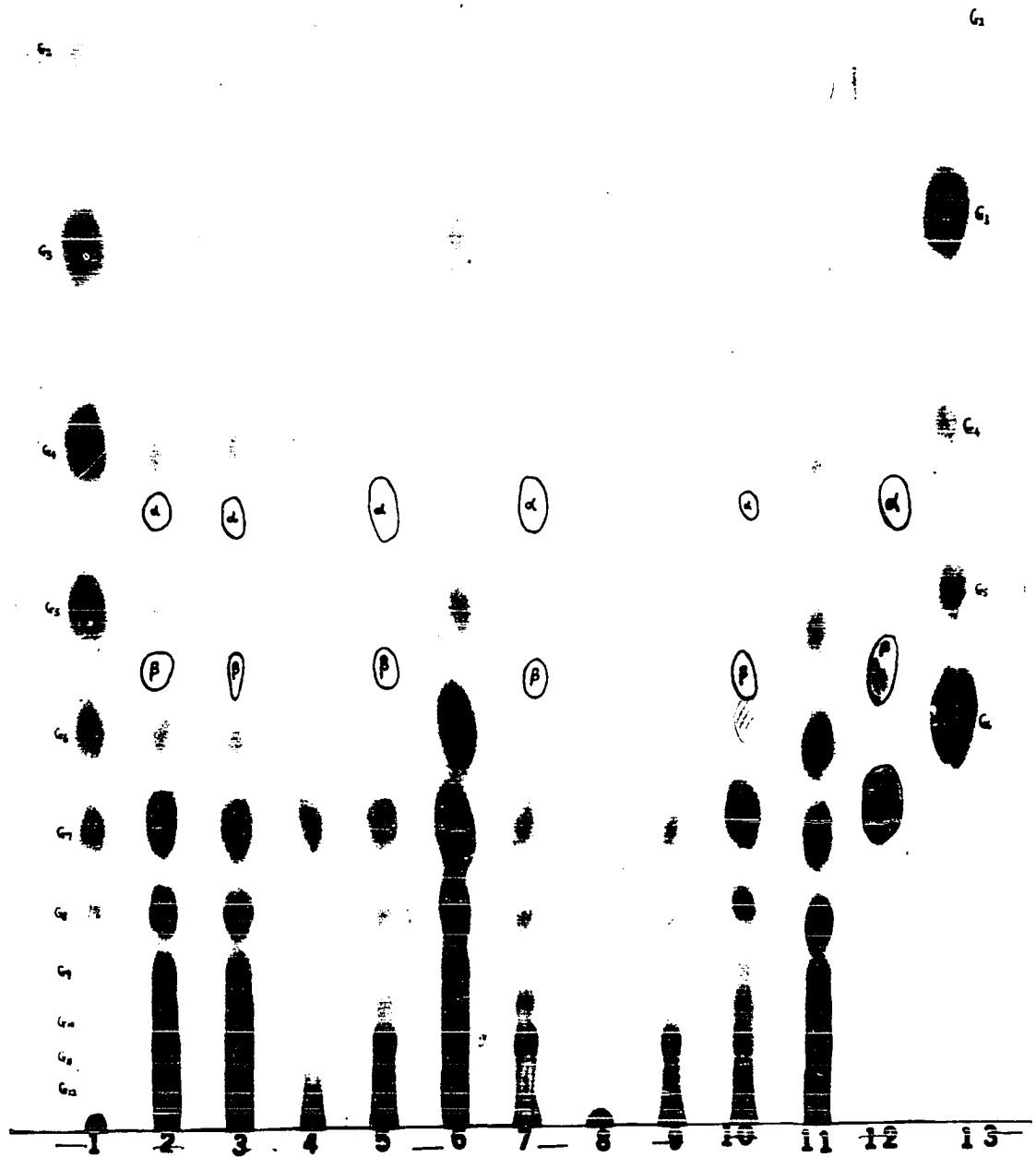
2 and 3 are the enzyme preparation obtained after $(\text{NH}_4)_2\text{SO}_4$ and acetone fractionations respectively

4 and 6 represents protein fraction A (see Fig. 4)

5 and 7 represents protein fraction B (see Fig. 4)

8 is protein fraction C (see Fig. 4)

9, 10 and 11 are the protein fractions I, II and III respectively (see Fig. 3)



production of reducing sugars) is retained until the last step in the purification process (#6 in Fig. 13).

Mode of action of BMA

Action of BMA on UL-¹⁴C-amylose Of central interest in this investigation is the determination of the mode of action of BMA. The Schardinger dextrans which are the primary products of the enzymic action have been well characterized. However, very little is known about the mechanistic route which leads to their formation. It has long been speculated that the modes of attack of BMA and β -amylase on their substrates are analogous. The current view on this is that the enzyme attacks the substrate from the non-reducing end, producing cyclic dextrans in much the same way that β -amylase produces maltose from the non-reducing end. This process is believed to continue, gradually shortening the chain, until the entire molecule has been converted into Schardinger dextrans. There is no definitive proof, whatsoever, for this mechanism. In view of recent findings by French *et al.* (88) and Abdullah and French (81) concerning the existence of branched cyclic dextrans in BMA starch digests and the successful purification of BMA, attention was therefore centered on the reexamination of the mechanistic details by which cyclic dextrans are formed.

For enzymic action on polymeric substrates, three possible modes of attack can be considered. The enzyme can degrade its substrate primarily by an endo mechanism producing a set of specific low molecular weight oligosaccharides as initial products in its action, or it could attack the substrate by an endwise action (exo mechanism) as explained above. A third

possibility would be the combination of these two mechanisms. The requirement of a free C₄ hydroxyl group in order to effect ring closure of its substrates demands that the mechanism of action of BMA be an exo type in producing the cyclic dextrans. If this is true, exposing high molecular weight linear substrate (*e.g.* amylose with DP (degree of polymerization) of about 2000 to the action of the enzyme should produce very little change in iodine color of amylose in the early stages of the reaction, since no significant reduction in the length of the amylose chains would occur. However, if the mechanism of action of the enzyme is endo, one would expect rapid decrease in the chain length of the polymeric substrate with consequent sharp decrease in iodine color.

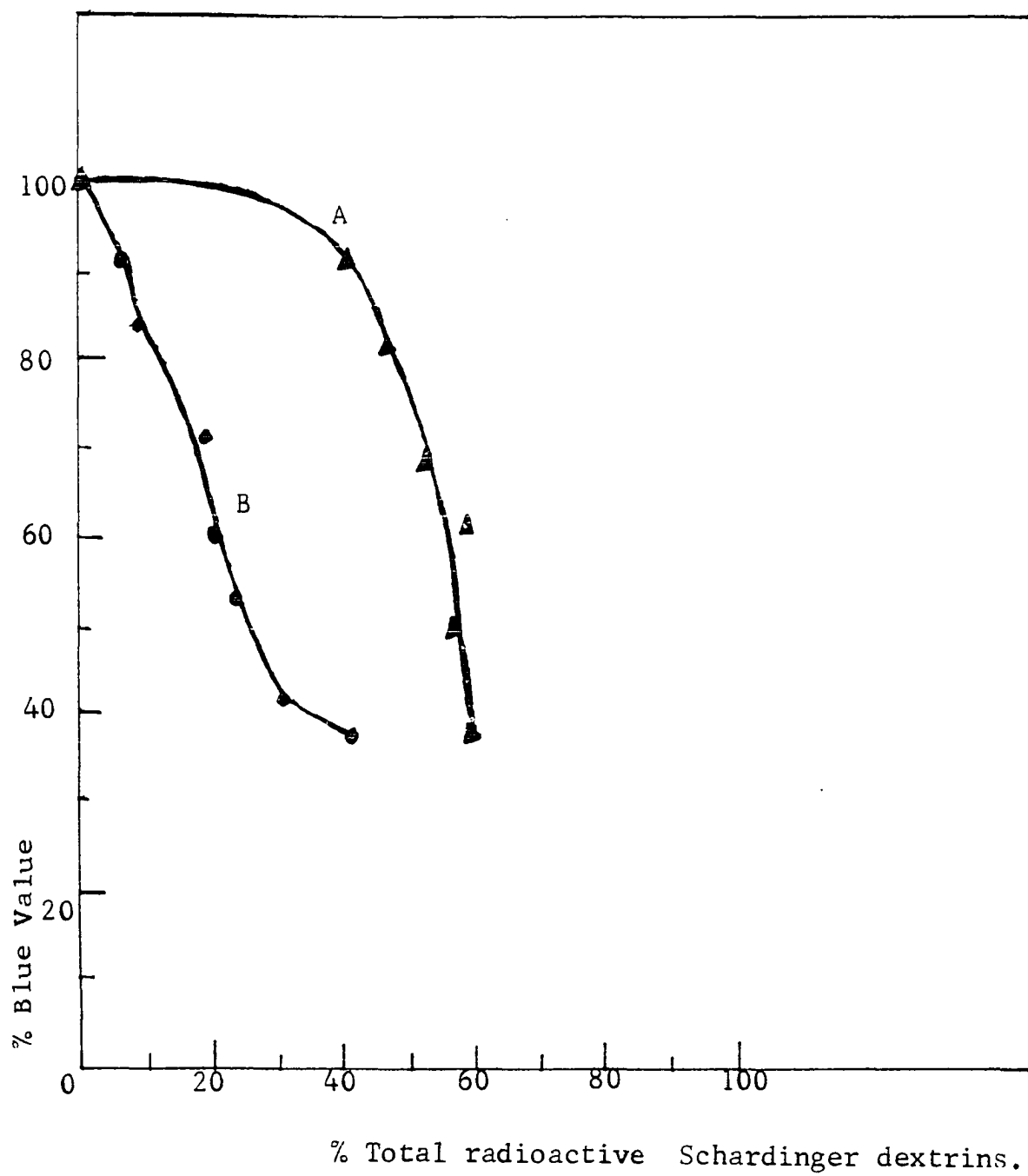
The first approach of getting some information on which of these mechanisms is employed by BMA was to follow the action of the enzyme on uniformly labeled ¹⁴C-amylose. Fig. 12 is a radioautogram showing the action pattern of BMA on ¹⁴C-amylose. The reference compounds are indicated as R₁ and R in Fig. 12, and the arabic numerals indicate digest times in minutes. Based on the conclusions made from Figs. 8 to 10, it is inferred from the radioautogram of Fig. 12 that the predominant products are the cyclic dextrans. The lower members of the homologous series (α , β , and γ) are clearly resolved. Failure to observe any radioactive reducing sugars such as glucose, maltose, and maltotriose is clearly an indication that the enzyme is essentially free of any hydrolytic contaminant. Fig. 14 is a plot of the drop in iodine color (blue value) with the per cent total resolvable cyclic dextrans produced. Curve A is obtained when the labeled amylose of undetermined degree of polymerization was diluted with cold amylose (DP \approx 1800). Curve B is obtained when the cold amylose

Figure 14. Comparison of the drop in iodine color (blue value) with the increase in per cent total chromatographically resolvable cyclic dextrans for the action of BMA on UL-¹⁴C-amylose

Blue value is defined as $(A_t/A_0) \times 100$, where A_0 and A_t are the absorbancies (620 nm) of the iodine complex of the digest at zero time and at t minutes of reaction.

A - carrier amylose has DP 1800

B - carrier amylose has DP 200.



carrier has a DP of about 200. From the nature of curve B it is concluded that the mechanism of action of BMA is endo. The unusual relationship obtained in curve A is interpreted to mean a strong preference of the enzyme for smaller fragments. That is, if the labeled substrate is of shorter chain length than the carrier, then the enzyme reacts faster with these shorter segments than with the longer chains, thereby depleting them before depleting the larger molecules. The overall effect will be a gradual decrease in blue value (which measures rate of degradation of the carrier substrate) and a rapid increase in total radioactive cyclic products (*cf.* Fig. 14A). On this basis the data from Fig. 14B, therefore, implies an equal preference for both the radioactive substrate and the carrier, assuming that both are almost of identical length. This assumption seems justified because the radioactive substrate used in this study was prepared by extensive pullulanase action on UL- ^{14}C -starch with subsequent chromatography on Sephadex G-100. Pullulanase is specific for hydrolyzing α -1 \rightarrow 6 glucosidic linkages, and it is conceivable that the predominant linear products from such digest will be the exterior A and B chains of the amylopectin fraction of starch. These exterior chains are usually of moderate chain length. It is concluded from Fig. 14, therefore that the initial action of BMA on high molecular weight substrates is that of an endo attack to produce low molecular weight amyloextrins with the consequent rapid decline in blue value.

Action of BMA on amylo By analogy to the typical endoases (*e.g.* α -amylases) it might be expected that the initial action of BMA on amylose will result in production of new reducing ends. Further efforts in the determination of the mode of action of this enzyme was geared towards

Figure 15. Incorporation of tritium into BMA digest of amylo1

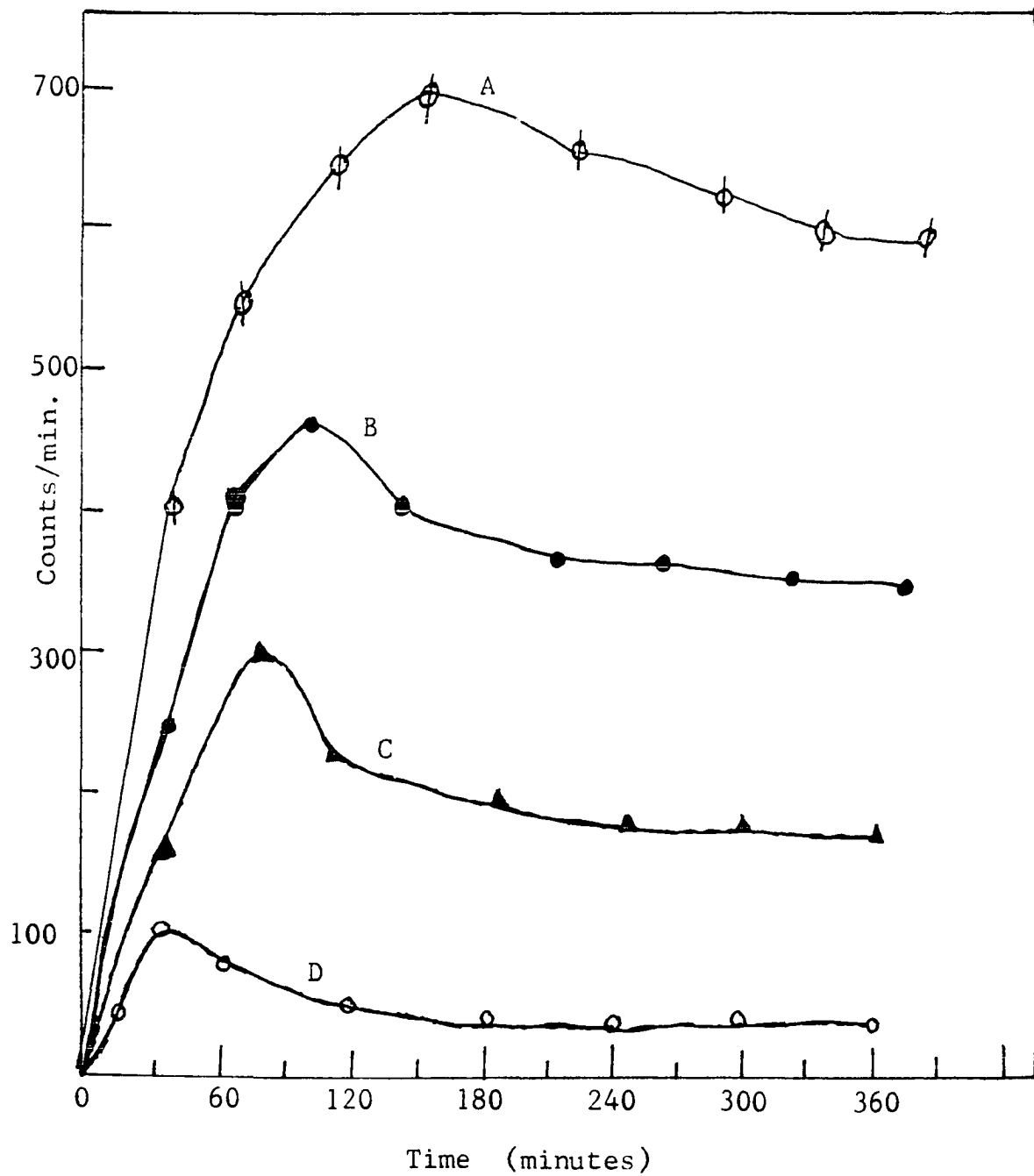
A - 2 mg amylo1/ml of reaction mixture

B - 1 mg amylo1/ml of reaction mixture

C - 0.75 mg amylo1/ml of reaction mixture

D - 0.1 mg amylo1/ml of reaction mixture

Enzyme concentration was maintained at 0.10 Tilden-Hudson
unit per milligram of substrate



detection of reducing products of the enzymic action. Tritium labeling studies on amylo1 was therefore carried out at various digest time and various substrate concentrations using sodium borotritide. If reducing ends were being formed by the enzyme, they would be reduced by sodium borotritide with the incorporation of tritium.

Fig. 15 shows the curve obtained when the amount of tritium uptake is plotted against the digest time. It is evident that there is a significant tritium incorporation which passes through a maximum and then decreases. The shift in the maximum with increase in substrate concentration indicates (on a relative basis), a decrease in the number of hydrolytic reactions. The occurrence of hydrolytic reactions would be expected to be concentration dependent. As the substrate concentration decreases (corresponding to a decrease in the number of end groups) probability of transfer to water as acceptor molecule is much increased. This would be reflected in a proportional increase in the rate of tritium incorporation and the shift to an early maximum observed with the decrease in substrate concentration. Fig. 16A depicts the elution profile of the sodium borotritide treated digest products on Sephadex G-50 fine. A gradual shift of the carbohydrate peaks towards high V_e/V_0 ratio is observed as the reaction progresses. This indicates significant reduction in the molecular size of the digest mixture, suggestive of the presence of low molecular weight reducing intermediates in the digest. The result in Fig. 16B shows that for a given digest the carbohydrate peak and radioactivity peak are not coincident. It can also be seen that the amount of carbohydrate that is radioactive is small.

Since the cyclic dextrin controls picked up radioactivity from

Figure 16. Gel filtration of sodium borotritide treated 0.2% amylo1-BMA digest on Sephadex G-50 fine

A - Total carbohydrate by phenol sulfuric acid method

Peaks at elution volume 96 ml are the cyclic dextrans

B - Rate of tritium incorporation in counts per minute

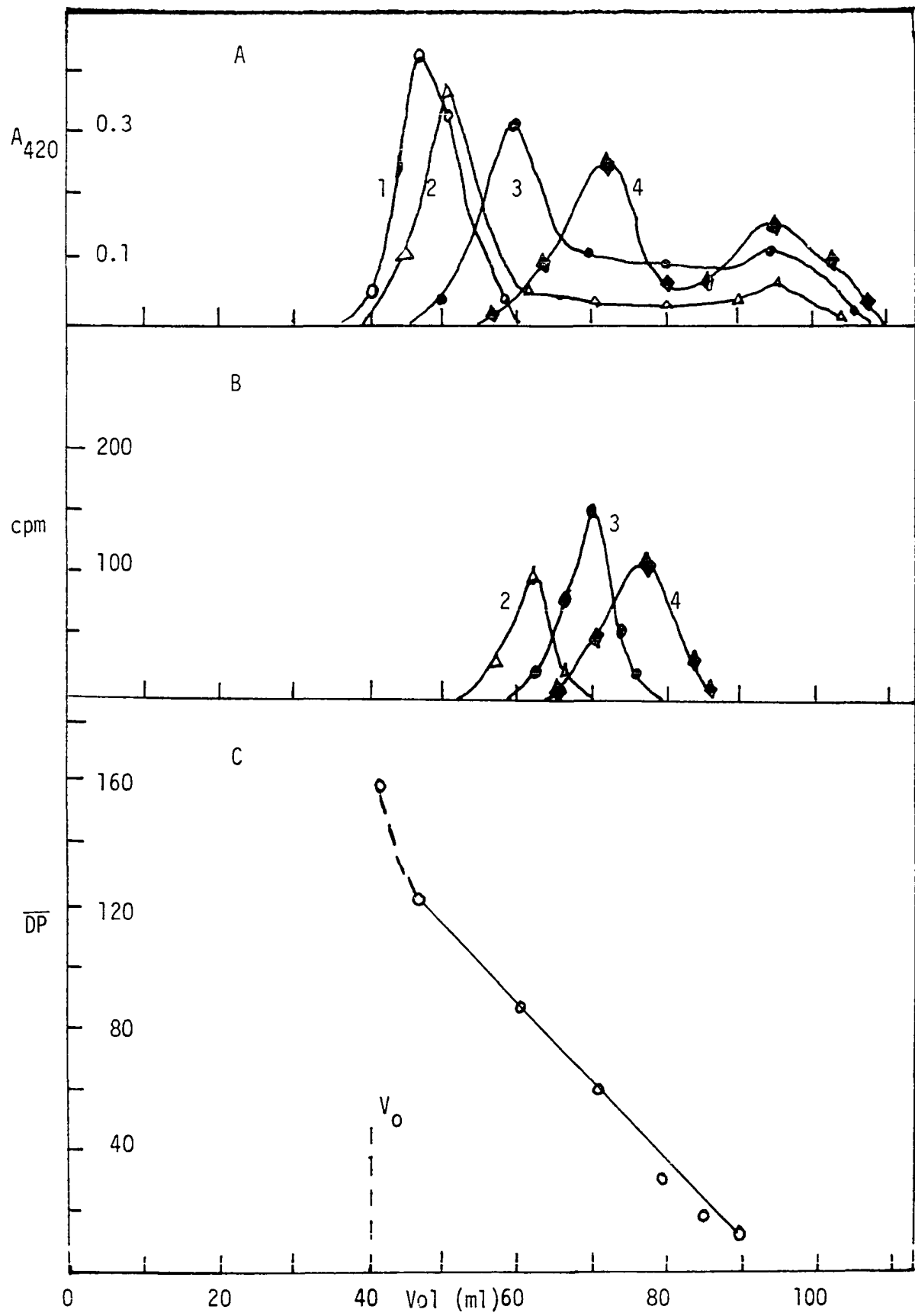
C - Estimation of the average DP of the reaction products

1 - Zero time digest

2 - 30 minutes digest

3 - 90 minutes digest

4 - 180 minutes digest



sodium borotritide treatment it was necessary to examine whether the radioactive products of the enzymic digest are indeed new reducing ends produced by enzymic action or whether they are experimental artifacts resulting from some anomalous effect of sodium borotritide on cyclic dextrans. From Fig. 16B it is observed that the sodium borotritide treated cyclic dextrans ($V_e = 96$ ml) do not migrate at the same position as the labeled products from the enzyme digest and that there was no production of radioactive cyclic dextrans in the BMA digest. It is, therefore, concluded that the radioactive compounds in the enzymic digest originate as a result of BMA action amylose.

Characterization of the concentrated radioactive fractions was accomplished by treating the fractions with β -amylase for 24 hours followed by paper chromatographic analysis. After sectioning and counting the radioactivity in a liquid scintillation spectrometer, about 85% of the radioactivity appeared as maltitol and 10% as maltotriitol. This strongly suggests that the radioactive compounds obtained in the BMA digests are linear molecules. The approximate sizes of these dextrans were determined from a calibration curve (see Fig. 16C). Although the nonlinearity of the calibration curve prevents precise determination of molecular size, it is estimated that the initial amylo-dextrans produced by BMA have an average DP of 140 glucose units.

From the data presented in Figs. 14-16, it is concluded that some of the reactions of BMA on large polymeric substrates is endo, although the formation of the cyclic dextrans essentially goes by an exo-mechanism. One possible explanation of how this could be accomplished is to imagine that the enzyme and substrate initially approach each other by a process

of random diffusion. As the substrate and enzyme diffuse about in solution, a collision occurs at the right orientation resulting in formation of a productive complex. This demands that the substrate molecule first hunt about over the surface of the enzyme for the proper binding site. In the next step, hydrolytic cleavage occurs and the two fragments obtained may (1) either diffuse away to form new enzyme-substrate complex that will undergo rapid degradation by an exo-mechanism to give cyclic dextrin or (2) one of the fragments diffuses away while the other realigns itself on the surface of the enzyme. This second realignment may lead to production of cyclic dextrans by an exo-mechanism.

SUMMARY

Bacillus macerans amylase has been isolated in reasonably high yields from a culture medium which utilizes amyloextrins ($DP \approx 44$) as the sole carbon source for the organism.

The enzyme has been purified to better than 300-fold and shown to be a single, homogeneous protein by disc gel electrophoresis.

The enzymic behavior of the purified BMA was investigated and the following observations reported:

1. The enzyme has an optimum pH range between 6.2 to 6.5.
2. It displays a diphasic Arrhenius plot with μ (activation energy) of 8.4 kilocalories per mole between 20°C and 55°C, and 4.56 kilocalories per mole between 55°C and 75°C.
3. The molecular weight as estimated by gel filtration chromatography is 50,000.

The action pattern of the purified enzyme was studied by descending paper chromatography. Results showed that the enzyme produces predominantly cyclic dextrans from starch, amylose, amylopectin, and glycogen. No detectable reducing sugars were observed on any digests of this enzyme with polymeric substrates. The mode of action of the pure enzyme was investigated. Evidence is presented to support the conclusion that the mechanism of action involves a significant initial endo attack on amylose producing amyloextrins thereby, and a rapid conversion of the amyloextrins into cyclic dextrans by an exo attack.

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